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ESTIMATING LETHAL AND SEVERE TOXIC EFFECTS IN MINIPIGS FOLLOWING 10, 60, AND 180 MINUTES OF WHOLE-BODY GB VAPOR EXPOSURE

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14. ABSTRACT
Sexually mature male and female Gottingen minipigs were exposed to various concentrations of GB vapor via whole-body inhalation for 10, 60, or 180 min. Signs of nerve agent exposure were classified as lethal, severe, or moderate. Maximum likelihood estimation was used to calculate the median effect levels: lethal (LCT_{50}) and severe (ECT_{50}) for each gender-duration combination. Ordinal regression was used to model the product of concentration and time profile of the agent toxicity. Contrary to the values predicted by Haber's rule, LCT_{50} and ECT_{50} values increased as the duration of the exposures increased. The values for LCT_{50} (with 95% confidence limits) for 10-, 60-, and 180-min exposures in male minipigs were 72.5 (57.3-91.6), 105.7 (85.6-130.6), and 182.3 (145.2-228.9) mg.min/m^3, respectively. The LCT_{50} values (with 95% confidence limits) for 10-, 60-, and 180-min exposures in female minipigs were 86.9 (69.2-109.2), 127.1 (100.7-160.4), and 174.3 (134.7-225.5) mg.min/m^3, respectively. The data were best fit using a probit slope of 15.7 and toxic load exponent of 1.38 (95% confidence limits of 1.25-1.51). Although males were significantly ($p = 0.01$) more sensitive than females to the lethal effects of GB vapor, the ratios of lethal to severe concentrations were higher in female minipigs (99% ANOVA confidence), indicating that there is less difference between severely toxic and lethal dosages in the female as compared to male pigs.

15. SUBJECT TERMS

Duration	Concentration	Lethality	Inhalation	Sarin
Minipig	Low-level	Gottingen	Swine	GB

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PREFACE

The work described in this report was authorized under Project No. 206023, Low-Level Toxicology. The work was started in June 2003 and was concluded in January of 2004. The experimental data is contained in laboratory notebook 02-0109 and on compact discs. Raw data and the final report from this study will be stored in the Toxicology Archives, Building E-3150, Aberdeen Proving Ground, MD.

In conducting this study, investigators adhered to the "Guide for the Care and Use of Laboratory Animals," National Institutes of Health Publication No. 86-23, 1985, as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, Washington, D.C. These investigations were also performed in accordance with the requirements of AR 70-18, "Laboratory Animals, Procurement, Transportation, Use, Care and Public Affairs," and the U.S. Army Edgewood Chemical Biological Center Institutional Animal Care and Use Committee (IACUC), which oversees the use of laboratory animals. This project's assigned IACUC protocol No. 02-341 was approved on 6 August 2002.

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ESTIMATING LETHAL AND SEVERE TOXIC EFFECTS IN MINIPIGS FOLLOWING 10, 60, AND 180 MINUTES OF WHOLE-BODY GB VAPOR EXPOSURE

1. INTRODUCTION

Throughout history, research studies on nerve agents, have been conducted on all possible routes of human exposure: intravenous, percutaneous, intramuscular, subcutaneous, intragastric, and inhalation. While these studies provide valuable basic information on nerve agent intoxication, accurate extrapolation from one route of entry to another is difficult. Additionally, the progression of toxic signs via one exposure route may be different from another. For instance, in an inhalation exposure of sarin (GB) vapor, constriction of the pupils is the first noticeable sign of exposure while in GB exposures by the subcutaneous or intravenous routes, pupil constriction occurs well after the other signs of exposure or occurs irregularly.[1] The likely routes of exposure for the warfighter in a battlefield situation would be limited to percutaneous and inhalation. Percutaneous exposure is possible through contact with contaminated equipment or aerosol created from an attack. However, in such an attack, inhalation of nerve agent vapor would be the primary route of exposure. If GB vapor were used, the chances of a percutaneous exposure are slim. GB has a vapor pressure of 2.9 mm^ Hg at 25 °C and is likely to evaporate long before it can be absorbed through the skin. Indeed, open air testing has demonstrated that GB has minimal effectiveness in humans via the percutaneous route of exposure.[2] Therefore, the primary hazard posed by GB is through inhalation.

Unfortunately, the safety and logistics of performing an inhalation study have been limiting factors on the type of data collected. Historically, data collected during an exposure have been subjective and limited to clinical observations. Additionally, most of the data belonged to the post-exposure time period; therefore, physiological changes occurring during a whole-body inhalation exposure have been insufficiently documented. The methods described by Hulet et al. have made it possible to collect data during a real time inhalation GB exposure.[3]

Traditionally, the military and other organizations dealing with inhalation toxicology have accepted Haber's principle of dosage, the product of concentration (C) and time (T), as constant over time when assessing the impact of nerve agent vapor exposures.[4] Haber's rule was used to extrapolate dose/response data (based upon relatively short exposure times) to predict response probabilities involving longer exposure times. However, this concept is now considered inadequate for assessing the biological effects of exposure to many acutely toxic gases and aerosols.[5] Recent efforts[3,6,7] have resulted in data including low concentration exposures over long periods, which can best be described with a toxic-load model.[5] For even a clear toxicological endpoint as lethality, historical assumptions used to extend the prediction of exposures over time have been shown to be overly conservative for GB, which is the best-studied agent.

In the toxic-load model, the median effective dosage (ECT_{50}) increases with exposure time in a non-linear relationship and the data can be fit to the toxic load equation,

$$C^nT = k.$$

C is the concentration,
T is the exposure duration, and
n is the toxic load exponent, which is dependent on the vapor or exposure scenario.

To develop models for predicting the probability of toxicity from low-level nerve agent exposures for different concentrations and durations of exposure, additional data from a non-rodent species are needed. Pigs have been found to be similar in anatomy and physiology to humans.[8] The intent of the current studies is to estimate the lethal concentrations of the nerve agent sarin (GB) as a function of exposure-duration in the Gottingen minipig. These results helped determine whether LCT_{50} and ECT_{50} (severe) values change with time, in the minipigs as was found in previous studies with rats.[9]

2. MATERIALS AND METHODS

2.1 Gottingen Minipigs.

Male and female Gottingen minipigs were obtained from Marshall Farms USA (North Rose, NY). Upon arrival at the testing facility, the minipigs underwent an initial health examination by the attending veterinary staff. The pigs were then quarantined for at least three days. After this time the involved research personnel familiarized the pigs to various procedures that included daily handling, change of location within the animal facilities and adaptation to a sling apparatus. While the animals were in their cages their existence was enriched by human interaction and unfettered access to play toys (hanging chains, bunny balls) or food treats on a daily, rotating schedule.

2.2 Surgical Procedure.

A more thorough description of surgical procedures can be found in Hulet et al, 2006.[3] Briefly, the surgical site (lateral neck from mandible to shoulder and mid dorsally between the shoulder blades of a minipig) was prepared for aseptic surgery by close-clipping the area and applying a surgical scrub (chlorhexidine or povidone-iodine) followed either by an application of isopropyl alcohol or a sporicidal agent. The surgical area was then covered with sterile drapes and the minipig positioned for surgery on a heated surgical table. The animal was evaluated throughout the procedure using an EKG monitor, temperature probe, pulse oximeter and respiratory monitor.

A silicone catheter (Bard access systems, 6.6 or 9.6 Fr.), impregnated with heparin and antimicrobial agent, was implanted in an external jugular vein and advanced to the anterior vena cava or right atrium. A subcutaneous tunnel, extending from the surgical site adjacent to the jugular vein to the exit site in the dorsal midline, was created with a hollow stainless-steel rod. The catheter was filled with sterile heparin saline (1%), grasped and pulled through from the dorsum to the ventral neck incision with at least 6 in. of catheter remaining external to the skin. The catheter position was adjusted so that blood samples could be readily

obtained. The catheter was secured by tying at least 2 sutures around the vein. A loop of catheter leading from the vein was also secured to the subcutaneous tissues using sutures. Once the catheter was appropriately adjusted, it was secured at the dorsal exit site and the incisions closed. The catheter was locked with 1% sterile heparin saline. Antibiotic ointment was placed on both incisions. Postoperatively, the minipig was given analgesics (buprenorphine 0.01 - 0.05mg/kg, BD) for at least 24 hr and subsequently, if indicated.

The minipigs were allowed at least 3 days for recovery from the surgical implantation of the indwelling catheters before being used for exposure to nerve agent vapor. During the waiting period, the vascular access ports on the pigs were flushed with heparinized saline, as needed. During the agent exposures, the catheters were maintained by a continuous intravenous infusion of lactated Ringers solution.

2.3 Blood Sample Collection.

"Real time" blood samples were drawn via the indwelling jugular catheters to assess cholinesterase inhibition and GB uptake via regeneration assays.[10] Blood samples were taken just prior to exposure and periodically throughout: approximately every 2 min during the 10-min exposure, every 15 min during the 60-min exposure, and every 20 min during the 180-min exposure. The total volume of blood drawn did not exceed 1% of the body weight of a minipig over a 1-week span. The drawn sample volumes were replaced by an equivalent volume of Lactated Ringers.

Assays for AChE and BChE activity were performed on whole-blood samples. Ten uL of whole blood was added to a disposable borosilicate glass tube (Chase Scientific Glass, Rockwood, TN) containing 2000 uL of distilled water. Two hundred uL of 0.69 mM phosphate buffer at pH 7.4 (EQM Research, Cincinnati, OH) was then added to each tube. The tubes were vortexed and allowed to sit at room temperature for 20 min. Two hundred uL of the sample solution from each tube was transferred to individual wells on a 96-well plate. Twenty-five uL of 30 mM 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) was added to each well. The plate was covered, and incubated at 37 $^{\circ}$C for 15 min.

For the determination of AChE activity, 25 uL of a solution containing 10-mM acetylthiocholine and 200-uM10-(α-diethylaminopropionyl)-phenothiazine, a specific inhibitor of butyrylcholinesterase (EQM Research, Cincinnati, OH), was added to the appropriate wells of the 96-well plate. For the determination of BChE activity, 25 uL of a solution containing 20-mM butyrylthiocholine (EQM Research, Cincinnati, OH) was added to the appropriate wells of the 96-well plate. The plate was shaken briefly to ensure mixing of the reagents and read at 450 nm and 37 $^{\circ}$C using a SpectraMax Plus microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) for 10 min and analyzed using SoftMax Pro LS version 4.3 software. AChE and BChE activity values were expressed as units of activity per mL of whole blood (U/mL).

2.4 Inhalation Chamber.

The minipig whole-body exposures were conducted in a 1000-L dynamic airflow inhalation chamber. The Rochester style chamber was constructed of stainless steel with glass or

Plexiglas windows on each of its 6 sides. The interior of the exposure chamber was maintained under negative pressure (0.25-0.30" H_2O), which was monitored with a calibrated magnehelix (Dwyer, Michigan City, IN). A thermoanemometer (Model 8565, Alnor, Skokie, IL) was used to monitor chamber airflow at the outlet.

Two sampling methods were used to monitor and analyze the GB vapor concentration in the exposure chamber. The first method was a quantitative technique using solid sorbent tubes (Tenax/TA) to trap GB, followed by thermal desorption. The second method was a continuous monitoring technique using a phosphorus monitor (HYFED, Model PA260 or PH262, Columbia Scientific, Austin, Texas). Output from the HYFED monitor provided a continuous strip chart record of the rise, equilibrium, and decay of the chamber vapor concentration during an exposure.

All air samples were drawn from the middle of the chamber and solid sorbent tube samples were drawn after the chamber attained equilibrium (t_{99}). The HYFED monitored the GB vapor concentration during the entire run. Solid sorbent tube samples were drawn from the chamber approximately every 10 min with each sample draw lasting 1 to 5 min depending upon chamber concentration and duration of exposure. All sample flow rates for the solid sorbent tube systems were controlled with calibrated mass flow controllers (Matheson Gas Products, Montgomeryville, PA). Flow rates were verified before and after sampling by temporarily connecting a calibrated flow meter (DryCal®, Bios International, Pompton Plains, NJ) in-line to the sample stream. Physical parameters (chamber airflow, chamber temperature, and relative humidity) were monitored during exposure and recorded periodically.

2.5 Solid Sorbent Tube System.

The automated solid sorbent tube sampling system consisted of four parts:

(1) A heated sample transfer line,
(2) A heated external switching valve,
(3) A thermal desorption unit, and
(4) A gas chromatograph (GC).

A steel sample line (1/16" o.d. x 0.004" i.d. x 6' length) extended from the middle of the chamber to an external sample valve. The sample line was commercially treated with a silica coating (Silicasteel® Restek, Bellefonte, PA) and covered with the heated (60 °C) sample transfer line (CMS, Birmingham, AL). The combination line coating and heating minimized nerve agent absorption onto the interior surfaces of the sample line. From the transfer line, the sample entered a heated (125 °C) 6-port gas-switching valve (UWP, Valco Instruments, Houston, TX). In the by-pass mode, vapor from the chamber continuously purged through the sample line and out to a charcoal filter. In the sample mode, the gas sample valve redirected nerve agent vapors from the sample line to a Tenax TA/Haysep sorbent tube (60-80 mesh) located in the thermal desorption unit (ACEM-900, Dynatherm Analytical Instruments, Kelton, PA). Temperature and flow programming within the Dynatherm desorbed nerve agents from the sorbent tube directly onto the GC column (RTX-5, 30 m, 0.32 mm i.d., 1-mm thickness), which was then followed by flame photometric detection (FPD - phosphorus mode).

The solid sorbent tube sampling system was calibrated by the direct injection of external standards (GB μg/ml) into the heated sample line of the Dynatherm. This way, injected nerve agent standards were put through the same sampling and analysis stream as the chamber samples. A linear regression fit ($r^2 = 0.999$) of the standard data was used to compute the GB concentration of each chamber sample.

2.6 Chemicals.

Isopropyl methyl phosphonofluoridate (GB) was used for all the vapor exposures in this study. Chemical agent standard analytical reagent material (CASARM)-grade GB 2035 (lot # GB-U-6814-CTF-N) was verified (usually 98.3 + 0.48 wt. % pure as determined by quantitative ^{31}P-NMR) and stored in sealed ampoules containing nitrogen. The ampoules were opened as needed either to prepare external standards or to be used as neat agent for vapor generation. All external standards for GB vapor quantification were prepared on a daily basis. Triethylphosphate (99.9% purity), obtained from Aldrich Chemicals, Milwaukee, WI, was used as the internal standard for the GB purity assays.

Analysis for agent impurities was conducted using acid-base titration as well as Gas Chromatography/Mass spectrometry (GC-MS) and ^{1}H NMR. Acid-base titration has been found to show the following impurity percentages based on mole ratios:

GB ANALYSIS

Compound	Mole %	Calculated Wt %
Methylphosphonofluoridic acid (Fluor Acid)	0.3	0.2
Diisopropyl methylphosphonate (DIMP)	0.2	0.3
Methylphosphonic difluoride (DF)	0.2	0.2

GC-MS positively identified DIMP, Diisopropyl phosphonofluoridate, Tributylamine, and Isopropyl ethylphosphonofluoridate, but did not quantify the amounts. Tributylamine was also confirmed using ^{1}H NMR with a concentration of < 0.1 weight% of GB.

2.7 Vapor Generation.

Saturated GB vapor streams were generated by flowing nitrogen carrier gas through a glass vessel (multi-pass saturator cell) that contained liquid GB. The saturator cell consists of a 100 mm long, 25-mm o.d. cylindrical glass tube with two (inlet, outlet) vertical 7-mm o.d. tubes connected at each end. The main body of the saturator cell contains a hollow ceramic cylinder that serves to increase the contact area between the liquid nerve agent and the nitrogen. The saturator cell allows nitrogen to make three passes along the surface of the wetted ceramic cylinder before exiting the outlet arm of the glass cell. The saturator cell body was immersed in a constant temperature bath so that a combination of nitrogen flow and temperature could regulate the amount of nerve agent vapor going into the inhalation chamber. The entire apparatus was contained within a generator box that was mounted at the top of the inhalation chamber. Typically, the saturator cell was loaded with 2 to 4 ml of liquid nerve agent (CASARM grade). To maintain the integrity of the liquid nerve agent within the cell, a

continuous low flow rate (1 to 2 ml/min) nitrogen stream was used. This setup was capable of precisely generating GB vapor over a concentration range of 0.001-2.0 mg/m^3.

2.8 Sling Apparatus.

A sling was used to restrain each minipig during the exposure to the GB nerve agent vapor. The frame of the sling was constructed of airtight stainless steel pipe and Swagelok™ fittings. The slings were custom designed (Lomir Biomedical, Inc., Malone, NY or Canvas and Awning supplies, White Marsh, MD) to fit the build and size of the minipigs. The body of each sling was made of canvas, which contained 2 holes on each side for legs so that it fitted comfortably around the pig. The sling also had two 2 straps that secured over the shoulders and hips. A muzzle harness was placed over the snout and secured both laterally and ventrally to the stainless-steel framing in order to prevent the pig from moving its head from side-to-side. The harness was fitted so that it did not interfere with the pig's ability to breathe.

2.9 Design and Data Analysis.

To determine the progression of experimental exposure concentrations, the up-and-down method with an assumed probit slope of 10 was used[11]. For this study, the binary response used for executing this method was dependent on the survival of the minipig for 24 hr after exposure to the nerve agent. The signs of nerve agent exposure were designated as moderate, severe, or lethal. A minipig was classified as having severe signs of exposure if it were gasping, prostrated, collapsed, or convulsing. Muscle tremors, salivation, lacrimation, or miosis constituted a moderate exposure.

The method of maximum likelihood estimation (MLE) was used on the resulting quantal-response data to calculate LCT_{50} values (and associated asymptotic 95% confidence intervals) and ECT_{50} values for severe signs for each of the 6-gender exposure- duration groups[12]. An example of an MLE calculation is presented in Appendix A of ECBC-TR-450.[3]

Up-and-down experiments normally use 6 to 10 subjects, definitely not enough to permit a reliable estimation of the probit slope. However, data from several up-and-down experiments can be combined to obtain enough animals (at least 30) to estimate the probit slope. The resulting dataset can then be analyzed via traditional probit analysis or ordinal logistic regression to obtain a probit slope estimate.[7,13,14, 15]

Equations 1 and 2 were used to model the response distribution:

$$Y_N = (Y_P - 5) = k_0 + k_C(\log_{10} C) + k_T(\log_{10} T) + k_s\,(Gender) + k_{TS}\,(\log_{10} T)(Gender) \quad (1)$$

$$Y_N = (Y_P - 5) = k_0 + k_C(\log_{10} C) + k_T(Time) + k_s\,(Gender) + k_{TS}\,(Time)(Gender) \quad (2)$$

where Y_N is a normit; Y_P is a probit; the k's are fitted coefficients; C is vapor concentration; and both T and Time represent exposure-duration. In eq 1, exposure-duration is treated as a covariate (T), whereas in eq 2, exposure-duration is treated as a 3-level factor (Time). The constant, k_{TS}, has 6 values, one for each Time-Gender combination. The constants, k_C and k_T,

are the probit slopes for concentration and Time, respectively. The toxic load exponent, n, is the ratio k_C/k_T. If this ratio is not different (with statistical significance) from 1, then Haber's rule is appropriate for modeling the toxicity. Otherwise, the toxic load model (C^nT) is the proper approach, assuming that there is no significant curvature in the data used to fit the model. Should significant curvature exist, the toxic load model is not appropriate, but it is still superior to Haber's rule in modeling the data.

The present protocol has exposure durations of 10, 60, and 180 min. For each of the exposure durations, 6 or 7 minipigs of each gender were used. Statistical analysis routines, contained within Minitab® versions 13 and 14 (Minitab, Inc., State College PA), and an in-house developed spreadsheet program were used for the analysis of the data.

3. RESULTS

3.1 Animals.

Thirty-eight pigs (19 male and 19 female) were exposed to concentrations of GB vapor to estimate LCT_{50} and ECT_{50} (severe) values. An additional male pig was used as an air control. At the time of the surgeries, the 20 males (19 experimental plus 1 control), weighed an average of 10.68 kg ± 0.26 (SEM) kg and the 19 females, weighed an average of 10.62 ± 0.21 (SEM) kg.

3.2 Median Effective and Median Lethal Dosages.

The results of the exposures were classified as moderate, severe, or lethal (see section 2.9 for a description of criteria). The ordinal scores are listed in Table 1. The method of maximum likelihood estimation (MLE) was used to calculate ECT_{50} (severe) and LCT_{50} values (and associated asymptotic 95% confidence intervals) for each of the 6-gender exposure-duration groups.[12] MLE calculations are shown in Appendix A. The EC_{50} and ECT_{50} (severe) values, with their respective 95% confidence intervals, can be found in Table 2 and are plotted in Figures 2 and 4. The ECT_{50} values, like LCT_{50} values, are not constant over time. The ratio of ECT_{50} (severe) values to LCT_{50} values are shown in Table 3. The ratio of lethal to severe concentrations was higher in female pigs (99% confidence).

3.3 Statistical Models for the Probability of Lethality.

To model the probability of lethality as a function of exposure concentration, exposure-duration, and gender, several models were fit to the quantal data shown in Table 1. The number of pigs used for each gender exposure-duration group was not large enough to estimate the response distribution. Instead, the response distribution was estimated using either eq 1 or 2 with the data for all 38 pigs (see Section 2.9). Ordinal regression was used to fit various response models (eqs 1 and 2) to the data. Appendix B contains printouts of the MINITAB® results.

Pig 63 may be considered an outlier. Tables 4 (analyses without pig 63) and 5 (analyses with pig 63) give the probit slopes and toxic load exponents obtained from various ordinal regression model fits. The recommended best model fit is model L5 (without pig 63):

$$Yn = \text{constant} + 12.4\ \log10(C) + 9.0\ \log10(T) - .605\ \text{Sex}$$

where the constant depends on the effect (severe or lethal) and Sex is coded as 1 for male and –1 for female.

The value of the toxic load exponent ($n = kC / kT$) was essentially independent of the model used. The toxic load exponent of model L5 was 1.38 with a 95% confidence interval of 1.24 to 1.52 when pig 63 was excluded (if pig 63 was included, $n = 1.37$ with 95% confidence interval of 1.20 to 1.53). Because this interval did not overlap one, Haber's rule was not considered an appropriate time dependence model for this dataset. Potential curvature in the data was evaluated by inserting a $(\log T)^2$ term into the model. This term was found to be statistically insignificant, regardless of the inclusion or exclusion of pig 63 in the analysis.

For executing the up and down method in this study, the probit slope on concentration, kC, was assumed to be 10 (see Section 2.9). The probit slope of the best model fit (L5) was 12.4 with a 95% range of 6.2 to 18.6 when pig 63 was excluded (if pig 63 were included, the probit slope would be 9.2 with a 95% range of 4.4 to 14.0). However, regardless of the inclusion of pig 63, all the 95% confidence intervals for the probit slope from the 6 model fits overlapped 10.

3.4 Gender Differences.

The models were tested for possible gender effects and Sex was found to be a significant term ($p = 0.014$ in model L2 and $p = 0.013$ in model L5). When pig 63 was excluded from the analysis, male minipigs were noticeably more sensitive than females. Gender was not a statistically significant term ($p = 0.063$ in Model L2 and $p = 0.067$ in Model L5) when pig 63 was included in the analysis. The gender term was not statistically significant for any of the models where exposure-duration was treated as a covariate. The interaction of Sex with Time (Model L1) or Sex with logT (Model L4) was not statistically significant, regardless of the inclusion of pig 63. The failure to find statistically significant differences between the interactions of gender and exposure-duration may have been due to the low sample size ($n = 6$ to 7). In contrast, when gender was considered, regardless of exposure-duration, the sample size was much larger ($n = 38$).

3.5 Baseline AChE and BChE Activity.

Baseline activities of red blood cell acetylcholinesterase (AChE) and plasma butylcholinesterase (BChE) were assessed prior to nerve agent exposures. Of the 38 pigs that were exposed to GB (19 males and 19 females), blood samples could not be collected during exposure from 3 males and 2 females because their catheters lost patency between the day of surgery and the day of the experiment. However, the baseline activities of the male control pig were included for a total of 17 males and 17 females. Baseline AChE activities showed no

significant differences ($p = 0.286$) when comparing male and female minipigs. However, female minipigs showed significantly less ($p = 0.022$) baseline BChE activity as compared to male minipigs. In order to increase the total number of minipigs used for the baseline measurements, the data from the pigs used in the current studies were combined with baseline measurements taken from pigs in other ongoing studies to provide a total of 37 females and 44 males.[3] The significant difference between baseline male and female BChE activity was more pronounced ($p = 0.004$) while there was still no difference in baseline AChE activity ($p = 0.681$).

3.6 Depression of AChE and BChE Activity.

Depression of cholinesterase (AChE & BChE) activity was assessed during the GB exposures by collecting blood specimens through the jugular catheter. Of the 38 pigs that were exposed to GB (19 males and 19 females) blood samples could not be collected from 3 males and 2 females due to the loss of patency in the catheter between the day of surgery and the day of the experiment. For male pig no. 58, samples could only be collected after exposure had been concluded and the pig had been removed from the exposure chamber. Table 6 identifies the lowest AChE and BChE values during exposure and gives the absolute lowest values (during or after exposure) for each pig. AChE values were decreased to 8% or less of the baseline values before the conclusion of the exposures in 31 out of 32 pigs. There was very little subsequent depression in AChE activity after the conclusion of the exposures. In fact, AChE depression in 22 of 32 pigs had reached the absolute lowest value during the course of the exposure. Depression of BChE was variable but dropped below 50% of baseline in only one of the pigs.

3.7 Rate of AChE Depression and GB Uptake.

Only the blood samples collected during the exposure time were utilized to calculate the rate of AChE depression. The theoretical dosage that each pig was exposed to, up to the point that each blood specimen was drawn, could be calculated. The depression of AChE (expressed as a percent of baseline measurements) was then plotted versus exposure dosage ($mg.min/m^3$). The depression of AChE activity versus dosage was best modeled using a polynomial fit. The associated x, x^2 and r^2 values from the polynomial curve fits are found in Table 7. The rate of AChE depression for each pig at any exposure dosage could be ascertained by taking the instantaneous slope of the curve at any dosage along the X-axis. There were no significant statistical differences found between the rates of AChE depression in pigs that lived versus the pigs that died. This was the case whether all the pigs were considered regardless of exposure-duration or whether the groups were broken down into separate exposure-durations.

Using the same methods as described above, multiple pair wise comparison t-tests were utilized to test for significant gender differences between groups along a range of dosages (Table 8). When the minipigs were considered as a group, regardless of the duration of exposure, the rate of ChE depression was significantly different ($p = 0.043$) between males and females at a dosage of 7.0 $mg.min/m^3$ and slightly outside of statistical significance ($p = 0.054$) at 8.0 $mg.min/m^3$. At dosages lower than 7.0 $mg.min/m^3$, the statistical differences became more pronounced ($p = 0.027$ at $Ct = 5$, $p = 0.012$ at $Ct = 1$). In all the circumstances, the statistical differences between the rates of AChE depression in male pigs had steeper slopes than the

female pigs. When the groups of male and female pigs were further broken down by exposure-duration, there were no statistical differences at any dosage for the 180-min exposures. There were statistical differences between the male and female rates of AChE depression in the 60-min exposures at total dosages up to 30 mg.min/m^3 ($p = 0.016$) and for the 10-min exposures up to 20 mg.min/m^3 ($p = 0.039$).

Only those blood samples collected during the exposure time were utilized to calculate the rate of GB uptake (see Section 2.3), which was plotted versus exposure dosage (mg/min/m^3) for each pig. These curves were best modeled using linear fits. The associated x (slope) and r^2 values for the linear fits are found in Table 7. Multiple pair wise comparison t-tests were utilized to test for significant differences between groups (Table 9). There were no significant differences between genders when males and females were considered as groups regardless of exposure-duration. This was also the case when the groups were individually broken down to 10-, 60-, or 180-min exposure-durations. In contrast, there was a highly significant difference ($p = 0.004$) between the uptake rates of GB between animals that survived for 24 hr and animals that eventually died. Not surprisingly, the rate of uptake was higher in the animals that eventually died.

4. DISCUSSION

4.1 LCT_{50} Values.

The calculated LCT_{50} values for pigs in the current study are consistent with the notion that larger mammals (pigs, dogs, cats, monkeys) have lower threshold values than smaller animals (mice, rats, rabbits). Larger mammals are also more reflective of estimated LCT_{50} values in humans. For instance, the 10-min LCT_{50} values in mice, male rats and rabbits are 380, 231 and 115 mg.min/m^3.[6,16] In comparison, the calculated LCT_{50}s for 10-min GB exposures in monkeys and male cats are 74 mg.min/m^3 and 79 mg.min/m^3, respectively.[17,18] Crook et al calculated the LCT_{50} for a 10-min GB vapor exposure in pigs to be 34 mg.min/m^3.[19] The LCT_{50} for 10-min exposures in male and females pigs in the current study are 72.5 and 86.9 mg.min/m^3, respectively.

The approximate 2-fold difference in the LCT_{50} values in the two studies may be attributed to several variables. The Crook study used Yorkshire pigs instead of Gottingen minipigs. The age of the Yorkshire pigs was identified only by saying that they were "just recently weaned," as opposed to the sexually mature pigs in this study. The pigs in the Crook studies were exposed in groups of 4 or 5 and were allowed free movement within the chamber, while in the current study, the pigs were individually exposed, restrained, and immobile. Additionally, the methods for generating accurate concentrations and analytically verifying those concentrations are likely more reliable now than they were 50 years ago. Despite the differences in the LCT_{50} values between the current study and the study by Crook, the numbers from the studies done on pigs are considerably closer to human LCT_{50} estimates than the data obtained from the rodent studies.

Bide et al. recently suggested an estimate for a 10-min GB vapor exposure of 57 mg.min/m^3 in humans.[20] This estimate was extrapolated based on data taken from 38 historical animal studies involving 7 species (none being swine), regardless of gender. The overview took into account the minute volume (MV) to body weight (BW) ratio for each of the species. The calculated MV/BW ratio for humans was 0.223. The next closest MV/BW ratio of species used in the study was 0.328 for dogs. The MV/BW ratio of pigs was 0.225.[21] The calculated toxic load exponent for the large data set was 1.38. Interestingly, the calculated toxic load exponent for the current study was also 1.38.

The LCT_{50} reported for a 10-min GB vapor exposure in monkeys was 74 (62-87 F.I.) mg.min/m^3.[17] In the Cresthull study, monkeys demonstrating the toxicological signs of collapse and/or convulsions were categorized as "incapacitated" and then an ICT_{50} (incapacitation) value for the 10-min GB exposure was calculated. The ratio of the ICT_{50} to LCT_{50} was 0.89. In the current study the toxicological signs of collapse and/or convulsions (along with prostration and gasping) were utilized to characterize a pig as portraying "severe" signs of exposure. The ratio of ECT_{50} (severe) to LCT_{50} values in male pigs for 10-, 60-, and 180-min exposures were 0.71, 0.79, and 0.74, respectively. The ratio of ECT_{50} (severe) to LCT_{50} for female pigs for 10-, 60-, and 180-min exposures were 0.89, 0.89 and 0.84, respectively. Statistically, the ratios of severe to lethal concentrations were higher in female pigs (99% ANOVA confidence) than in male pigs.

The data suggest that there is less difference between severely toxic and lethal dosages in female as compared to male pigs. While the Cresthull study used both male and female monkeys, the breakdown of sexes only stated that "most of them were females" limiting the ability to compare the two studies in order to distinguish whether statistical gender differences exist in the severe to lethal ratios of other species. The current study is most likely the first to identify gender differences between the ratios of severe to lethal effects. Sommerville analyzed the data of Mioduszewski et al. to calculate an ECT_{50} (severe) to LCT_{50} ratio of 0.79 for a vapor GB exposure in rats.[9,15] This value was calculated for all of the animals taken together as a single group, regardless of the duration of exposure or gender. The rat data of Mioduszewski et al. is being reanalyzed to determine whether the data from this study are applicable to rats.[9]

Crook et al. determined that 87% of the pigs that died from the 10-min GB vapor exposures did so either during exposure or within the first 10 min after the conclusion of the exposure.[19] In the current study, 83% of the pigs that died from the 10-min exposure to GB did so within the first 10 min after exposure. Similarly, 72% of the pigs that died, regardless of exposure-duration, did so either during the exposure or within the first 10 min after the exposure. These data suggest that the toxic actions of GB occur because of the inhalation of GB vapor rather than the delayed absorption of GB through the skin. Indeed it has been demonstrated in open air testing that GB has minimal effectiveness in humans as a nerve agent via the percutaneous route of exposure.[2]

4.2 Gender Differences.

In 1998, a recommended change to airborne occupational exposure limits suggested that the Immediately Dangerous to Life or Health (IDLH) exposure guidelines for inhaled GB be lowered.[22] This suggestion was made to correct the failure of the existing guidelines to take into account that there may be differences in sensitivity to nerve agents based on gender. The existing value at the time for a 30-min exposure to GB of 0.2 mg/m^3 was lowered to 0.1 mg/m^3. This suggestion was made based on work done by Callaway and Blackburn in which female rats were found to be as much as twice as sensitive to the lethal effects of inhaled GB than male rats.[23] The significantly greater sensitivity to inhaled GB in female rats has subsequently been shown to occur over longer (240 min) durations of exposure as well.[6] The female hamster has also been identified as being more susceptible to GB vapor exposure than its male counterpart.[24] In contrast, male mice have been identified as being significantly more sensitive than female mice to GB vapor via inhalation[24,25] and intravenous administration.[26] Given that there are no relevant human data available and there is a surprising lack of literature investigating gender differences in sensitivity to inhaled GB in the higher species (cat, dog, pig, monkey), the best possible course of action is to base human estimates on available data, the majority of which are derived from rodents. However, the current study has identified that male pigs are significantly ($p = 0.01$) more sensitive to inhaled GB than female pigs. While this conclusion is not, by itself, enough to suggest that current human estimates be revised, gender differences in a species that more closely reflects human toxicity estimates warrants consideration, if not perhaps priority, in deriving such estimates.

4.3 Cholinesterase Depression and GB Uptake.

Thirty-one of the 32 pigs (97%) from whom the blood samples were collected during exposure showed decreases in AChE activity to 8% or less of baseline values by the end of the exposure-duration. Despite the low-levels of AChE activity, 16 of the 32 pigs survived. As early as 1958, Grob and Harvey identified that red blood cell cholinesterase activity could be depressed in humans to near zero (with multiple low dose exposures) without resultant death.[27] The current study supports the existence of a poor correlation between absolute AChE activity values and predictability of lethality. Additionally, there were no significant differences found between the rates of AChE depression in animals that lived versus animals that died.

Surprisingly, rates of AChE depression were significantly different between male and female pigs, with the males showing the steeper slopes. Although other literature sources have not yet been found to support this hypothesis, there are several possible explanations that could account for this finding. The three most likely are

1) Differences in the rate of uptake of the nerve agent into the systemic circulation,

2) Female pigs possessing an additional buffering capacity within the circulation, which acts as a "sink" to prevent binding of the nerve agent to the cholinesterase, and

3) Differences in the inherent characteristics of the red blood cell cholinesterase of the two sexes.

In addressing these possibilities, the evidence provided by the GB uptake rates makes differences in the breathing rates of the two sexes unlikely. No statistically significant differences were observed in GB uptake rates between the male and female pigs.

Solid evidence in other species, especially rodents, supports the idea of gender differences in enzymes with the potential to provide a buffering capacity. Female rats have higher plasma cholinesterase and carboxylesterase activities than male rats.[28-30] Female mice have two-fold higher plasma butylcholinesterase activities than male mice.[31] In human adults, females have been shown to have significantly higher baseline RBC AChE values, but significantly lower baseline BChE activities than males.[32,33] Additionally, there is evidence that RBC AChE activity can be cyclically regulated by hormonal influence in females.[34] In the present study, baseline AChE activities in male and female pigs show no statistical differences (see Section 3.5). However, baseline values of BChE were significantly higher in male pigs than in female pigs. The male pigs were significantly more sensitive to GB. Interestingly, in rats, females have higher BChE activity and they are also more sensitive to GB than their male counterparts. There is a lack of data available to determine if there is a gender derived nerve agent sensitivity difference in humans and if so which sex is more sensitive. It should be noted, however, that like male pigs and female rats, men have higher baseline BChE activities than women.[33]

Neither absolute AChE activity levels nor the rate of AChE activity depression were accurate measurements for predicting mortality. However, there was a significant difference in the rate of GB uptake in animals that lived versus animals that died. Not surprisingly, pigs that died had a significantly greater rate of GB uptake than those that survived. Since the rate of GB uptake is proportionate to the concentration of nerve agent, most likely the animals that died were exposed to higher concentrations. Nonetheless, the data suggest that the rate of nerve agent uptake is a more accurate predictor of toxicological endpoints in whole-body inhalation exposures with nerve agents than the current standard of analyzing depression of cholinesterase activity.

5. CONCLUSIONS

The current study was conducted with the intent of estimating lethal concentrations of the nerve agent sarin (GB) as a function of exposure-duration in the Gottingen minipig. Ordinal regression was used to fit various response models to the data. The ECT_{50} (severe) and LCT_{50} values were calculated in male and female pigs exposed to GB vapor for 10, 60, and 180 min. The value of the toxic load exponent was essentially independent of the model used. The toxic load exponent of the best-fit model (L5) was 1.38 (with a 95% confidence interval of 1.24 to 1.52). Because this interval does not overlap one, Haber's rule is not considered an appropriate time dependence model for this dataset. Potential curvature in the data was evaluated by inserting a $(\log T)^2$ term into the model and this term was found to be statistically insignificant. The probit slope of the best model fit (L5) was 12.4 with a 95% range of 6.2 to 18.6. The models were tested for possible gender effects and Sex was found to be a significant term ($p = 0.013$ in model L5), with males being significantly more sensitive than females. The ECT_{50} values (severe) were approximately 71 to 79 % of the LCT_{50} values in male

pigs and 84 to 89% of the LCT_{50} values in female pigs. The ratios of severe to lethal concentrations were higher in female minipigs (99% ANOVA confidence) indicating that there is less difference between severely toxic and lethal dosages in the female as compared to male pigs.

Baseline RBC AChE activities showed no significant differences when the results of male and female minipigs were compared. However, female minipigs showed significantly less ($p = 0.022$) baseline BChE activity than male minipigs. Depression of AChE and BChE activity (expressed as a percent of baseline measurements) was plotted versus exposure dosage ($mg.min/m^3$). The AChE activity was decreased to 8% or less of baseline before the conclusion of the exposures in 31 of the 32 pigs, with very little subsequent depression in AChE activity after the conclusion of the exposures. Depression of BChE was variable but dropped below 50% of baseline in only one of the pigs. There were no significant differences between the rates of depression of AChE activity in the pigs that lived versus the pigs that died. However, the rate of depression of AChE activity was significantly higher ($p = 0.043$) in male pigs than in female pigs at dosages below 7.0 $mg.min/m^3$. There was a highly significant difference ($p = 0.004$) between the uptake rates of GB between the pigs that survived for 24 hr after exposure and the pigs that eventually died. Not surprisingly, the uptake rate was higher in the animals that eventually died. The data suggest that the nerve agent uptake rate is a more accurate predictor of toxicological endpoints in whole-body inhalation exposures with nerve agents than the current standard of analyzing depression of cholinesterase activity.

Table 1. Durations and Concentrations of GB Exposure for Male and Female Pigs

Sex	Animal #	Time (minutes)	Concentration (mg/m^3)	CT ($mg.min/m^3$)	Result (1=moderate, 2=severe, 3=lethal)
Male	41	180	1.00	180.00	3
Male	42	10	9.40	94.00	3
Male	43	10	6.70	67.00	3
Male	44	10	9.50	95.00	3
Male	45	180	1.34	241.20	3
Male	46	60	1.68	100.80	1
Male	47	10	7.45	74.50	2
Male	48	60	2.00	120.00	3
Male	49	60	2.50	150.00	3
Male	50	180	0.90	162.00	2
Male	51	60	1.64	98.40	2
Male	52	180	1.10	198.00	3
Male	53	180	0.80	144.00	1
Male	54	10	5.35	53.50	1
Male	55	60	1.78	106.80	2
Male	57	60	1.90	114.00	3
Male	58	180	0.99	178.20	2
Male	59	10	5.90	59.00	2
Male	60	60	1.70	102.00	3
female	61	60	1.82	109.20	1
female	62	180	0.61	109.80	1
female	63	60	1.49	89.40	3
female	64	180	0.84	151.20	2
female	65	10	7.95	79.50	1
female	66	60	1.28	76.80	1
female	67	10	5.10	51.00	1
female	68	180	1.06	190.80	3
female	69	10	9.74	97.40	1
female	70	10	6.53	65.30	2
female	71	180	0.91	163.80	1
female	73	10	10.50	105.00	3
female	74	10	12.78	127.80	3
female	75	60	1.71	102.60	1
female	76	60	2.52	151.20	2
female	77	180	1.06	190.80	3
female	78	60	3.08	184.80	3
female	79	60	2.63	157.80	3
female	80	10	8.00	80.00	3

Table 2. MLE for Median Effective Concentrations and Dosages (with approximate 95% confidence intervals on the dosages)

Lethality

	Males			Females		
Exposure-duration (minutes)	**LC_{50}**	**LCT_{50}**	**95% Limits**	**LC_{50}**	**LCT_{50}**	**95% Limits**
10	**7.25**	**72.5**	**55.1—95.2**	**8.69**	**86.9**	**67.3—112.3**
60	**1.76**	**105.7**	**83.7—133.5**	**2.12**	**127.1**	**98.5—163.9**
180	**1.01**	**182.3**	**140.6—236.3**	**0.97**	**174.2**	**129.4—234.7**

Severe Effects

	Males			Females		
Exposure-duration (minutes)	**EC_{50}**	**ECT_{50}**	**95% Limits**	**EC_{50}**	**ECT_{50}**	**95% Limits**
10	**5.15**	**51.5**	**36.9—71.9**	**7.74**	**77.4**	**60.5—99.0**
60	**1.38**	**83.0**	**62.6—110.0**	**1.88**	**112.5**	**86.6—146.0**
180	**0.74**	**134.0**	**97.6—182.3**	**0.81**	**145.9**	**108.4—196.5**

Table 3. Ratios of ECT_{50} (severe) Values to LCT_{50} Values

Duration (min)	Gender	Severe / lethal	Gender	Severe / lethal
10	Male	0.711	Female	0.891
60	Male	0.785	Female	0.885
180	Male	0.735	Female	0.837

Table 4. Probit Slopes and Toxic Load Exponents (n) Obtained from Various Ordinal Logistic Regression Model Fits without Pig 63

ID	Terms in Model	k_C	SE(C)	k_T	SE(T)	n	SE(n)
L1	LogC Time Sex Time*Sex	15.7	4.0	---	---	---	---
L2	LogC Time Sex	12.6	3.2	---	---	---	---
L3	LogC Time	10.2	2.7	---	---	---	---
L4	LogC LogT Sex LogT*Sex	13.5	3.4	9.8	2.5	1.38	0.06
L5	LogC LogT Sex	12.4	3.1	9.0	2.3	1.38	0.07
L6	LogC LogT	9.9	2.5	7.2	1.9	1.37	0.06

Table 5. Probit Slopes and Toxic Load Exponents (n) Obtained from Various Ordinal Logistic Regression Model Fits with Pig 63

ID	Terms in Model	k_C	SE(C)	k_T	SE(T)	n	SE(n)
L1	LogC Time Sex Time*Sex	10.7	2.8	---	---	---	---
L2	LogC Time Sex	9.8	2.6	---	---	---	---
L3	LogC Time	9.0	2.5	---	---	---	---
L4	LogC LogT Sex LogT*Sex	9.9	2.6	7.2	1.9	1.37	0.08
L5	LogC LogT Sex	9.2	2.4	6.7	1.8	1.37	0.08
L6	LogC LogT	8.4	2.3	6.2	1.7	1.36	0.09

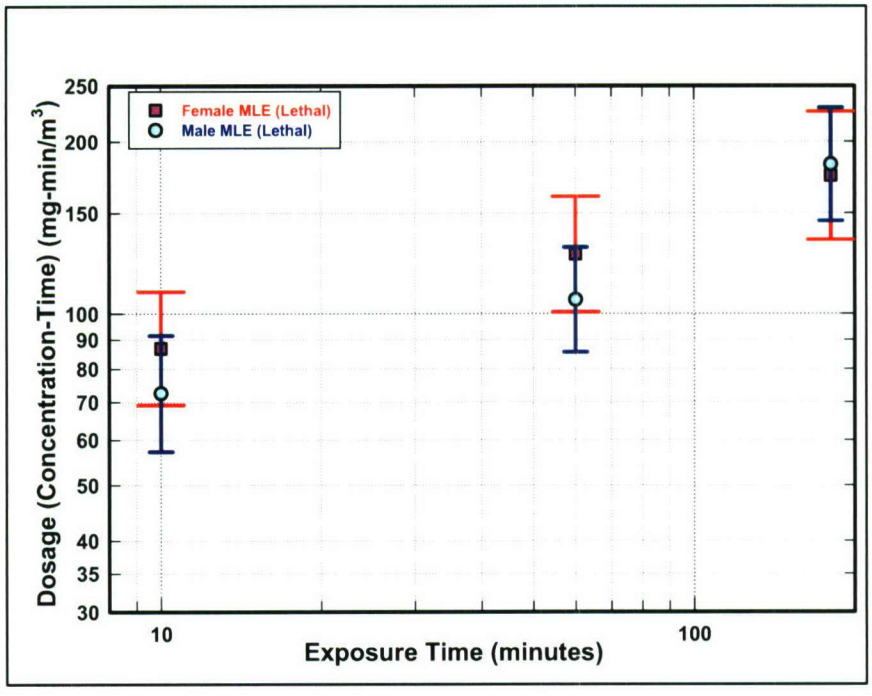

Figure 1. MLE LCT_{50} Estimates as a Function of Exposure Time for Male and Female Minipigs

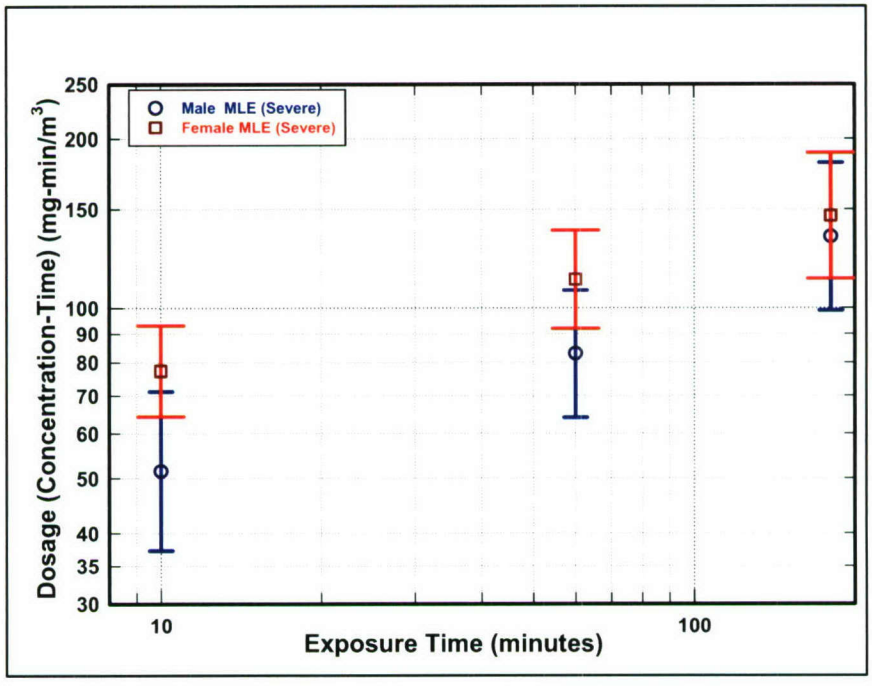

Figure 2. MLE ECT_{50} (Severe) Estimates as a Function of Exposure Time for All Male and Female Minipigs

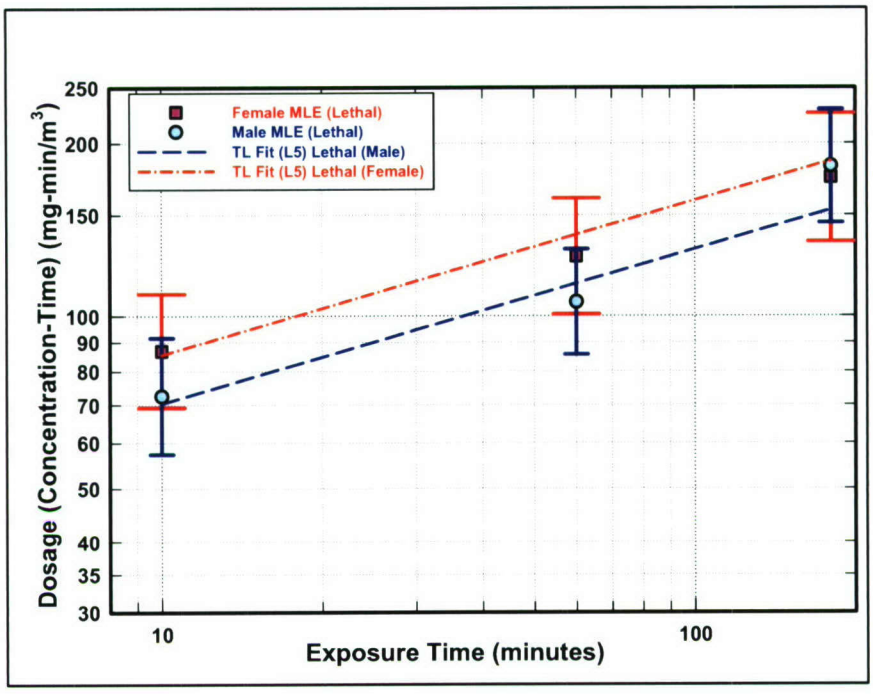

Figure 3. Toxic Load Fits (Model L5) of MLE LCT_{50} Estimates for all Male and Female Minipigs

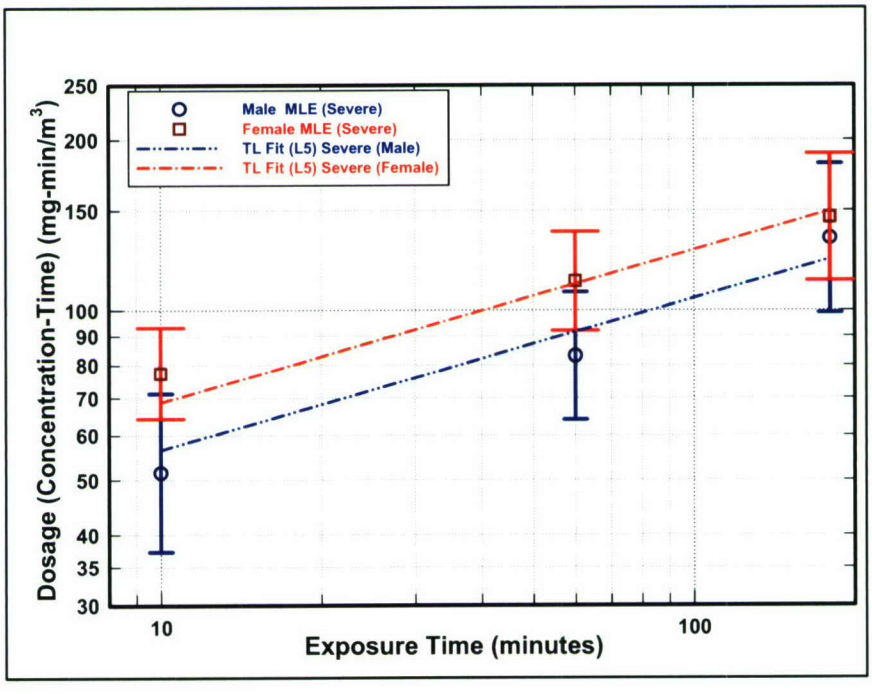

Figure 4. Toxic Load Fits (Model L5) of MLE ECT_{50} (Severe) Estimates for All Male and Female Minipigs

Table 6. Depression of AChE and BChE Activity

Pig #	Sex	low % AChE during	low % BChE during	% AChE lowest	% BChE lowest
67	f	13	100	6.5	100
63	f	4	80	4	74#
70	f	3	95	3	75
64	f	4	72	4	70
66	f	2	47	1	47
68	f	<1	69	<1	69#
61	f	7	82	4	78
65	f	7	95	4	90
69	f	8	99	4	94
74	f	5	74	4	61*
71	f	4	94	3	78
76	f	7	82	4	80
77	f	4	81	4	67*
78	f	7	81	7	70#
80	f	6	89	6	79*
79	f	3	85	3	74*
75	f	8	96	8	94
46	m	3	71	1	62
44	m	3	83	3	79*
45	m	1	75	1	75*
41	m	4	79	4	79*
42	m	3	84	3	71#
43	m	3	92	3	85*
48	m	3	68	3	68#
53	m	4	69	4	58
51	m	4	64	2	57
54	m	5	91	5	90
52	m	4	68	4	68*
55	m	6	91	6	91
59	m	4	88	4	85
57	m	3	70	3	70*
60	m	2	65	2	65*
58[a]	m	N.A.	N.A.	4	67

Summary table of AChE and BChE values at their lowest during the sarin exposure and the lowest value that was observed during or after the exposure. Pigs that died during the time of the exposure or within 10 min after the exposure are indicated by *. Pigs that died more than 10 min after the exposure concluded are indicated by #. [a]Blood samples were not able to be collected from pig 58 until the pig was removed from the chamber after the exposure.

Table 7. Depression Rate of AChE Activity and Uptake of GB

		RBC cholinesterase Depression (polynomial equation fits)			RBC GB uptake (linear equation fits)	
Pig #	Gender	x	x^2	r^2	x	r^2
80	F	-3.6994	0.0307	0.92	0.5036	0.9756
67	F	-2.1663	0.0071	0.99	0.1372	0.9416
70	F	-1.5801	0.0016	0.81	0.1820	0.9941
65	F	-2.0681	0.0105	0.99	0.2250	0.9818
69	F	-2.2803	0.0135	0.99	0.1371	0.9946
74	F	-2.1821	0.0122	0.93	0.3743	0.9607
77	F	-2.4840	0.0163	0.97	0.1377	0.9813
71	F	-1.4506	0.0063	0.91	0.0985	0.9654
68	F	-1.4246	0.0054	0.84	0.2009	0.9713
64	F	-1.6824	0.0080	0.93	0.1169	0.9745
63	F	-3.0448	0.0259	0.80	0.2724	0.9540
66	F	-2.7108	0.0197	0.98	0.1669	0.9940
61	F	-2.4151	0.0167	0.80	0.1704	0.8601
79	F	-1.7392	0.0081	0.89	0.1011	0.9553
76	F	-1.8013	0.0092	0.85	0.1019	0.8827
78	F	-1.3982	0.0054	0.91	0.2073	0.8460
75	F	-1.7762	0.0090	0.99	0.1367	0.9976
55	M	-2.5474	0.0168	0.94	0.2279	0.9733
57	M	-3.8283	0.0372	0.87	0.3182	0.9306
46	M	-2.4232	0.0165	0.90	0.1352	0.9701
48	M	-2.3165	0.0142	0.89	0.2484	0.9857
51	M	-2.7459	0.0199	0.93	0.2081	0.9687
60	M	-3.7345	0.0366	0.83	0.2669	0.9145
44	M	-3.0024	0.0234	0.86	0.2364	0.9908
42	M	-3.0258	0.0239	0.84	0.2277	0.9832
43	M	-4.2493	0.0465	0.88	0.2282	0.9694
54	M	-4.9404	0.0606	0.98	0.2220	0.9667
59	M	-2.9860	0.0202	0.97	0.1764	0.9762
52	M	-1.2620	0.0047	0.87	0.1671	0.9817
53	M	-1.6855	0.0840	0.90	0.1333	0.9925
45	M	-1.6755	0.0080	0.86	0.1413	0.9812
41	M	-3.1684	0.0280	0.78	0.2843	0.9755

Table 8. Pair Wise T-Test Comparisons of Rates of AChE Depression at Various Dosages. Statistical significance ($p < 0.05$) indicated by *.

t-test comparison	Dosage ($mg.min/m^3$)	P-value
Males vs. females - all	1	0.012*
Males vs. females - all	5	0.027*
Males vs. females - all	7	0.043*
Males vs. females - all	8	0.054
Males vs. females - all	10	0.088
Males vs. females - all	15	0.282
Males vs. females - all	20	0.696
Males vs. females – 10 min	5	0.025*
Males vs. females – 10 min	10	0.026*
Males vs. females – 10 min	15	0.028*
Males vs. females – 10 min	20	0.039*
Males vs. females – 10 min	25	0.128
Males vs. females – 60 min	5	0.041*
Males vs. females – 60 min	10	0.037*
Males vs. females – 60 min	20	0.027*
Males vs. females – 60 min	30	0.016*
Males vs. females – 60 min	35	0.071
Males vs. females – 180 min	5	0.947
Males vs. females – 180 min	10	0.670
Males vs. females – 180 min	15	0.514
Males vs. females – 180 min	20	0.432

Table 9. Pair Wise T-Test Comparisons of GB Uptake in to RBCs

t-test comparison	P-value
Male vs. female - all	0.470
Male vs. female – 10 min	0.552
Male vs. female – 60 min	0.068
Male vs. female – 180 min	0.340
Lived vs. died - all	0.004*

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APPENDIX A

BINARY AND ORDINAL PROBIT MODELS AND THE METHOD OF MAXIMUM-LIKELIHOOD ESTIMATION

A1.0 INTRODUCTION

Traditionally, median effective dosages are determined via the use of probit analysis.[1,2] In conventional probit analysis of binary response data, two parameters are estimated simultaneously from experimental quantal data using the method of maximum likelihood estimation (MLE):[3-8] the median effective stress, μ; and the standard deviation of effective stresses, σ. In toxicology, the effective stress is the base 10 logarithm of the effective dosage. Thus, the base 10 logarithm of the median effective dosage, $\log_{10}(ECT_{50})$, corresponds to μ, while the probit slope equals the inverse of σ. The probit model can also be extended for use with ordinal response data (categorical data that have three or more possible levels with a natural ordering [ex. mild, moderate and severe]).[1,3] For the ordinal probit model, there are individual μ's for each category, but the σ's for each category are assumed to equal each other.

The efficiency of the MLE procedure with the probit model is dependent on the sample size. Larger sizes provide unbiased and minimum variance estimates of both μ and σ, but this is not the case for small sample sizes. It has been shown that when solving for both μ and σ with small sample sizes that estimates for μ are unbiased (for all practical purposes), but estimates for σ are biased (with estimates for σ being too small on average).[3,8,9] Furthermore, the probability of MLE solution instability increases as sample size decreases when trying to solve for both μ and σ. Thus, for small sample sizes, a more pragmatic approach is commonly taken by fixing σ at some set value (based on historical knowledge of the system under study) while solving for μ. This is an underlying principle of the up-and-down method for estimating median effective stresses/dosages.[6]

The following are examples of both a binary and ordinal probit model applied to the male pig GB ordinal data (severe effects and lethality) for ten minutes exposures from the present study. The probit slope ($1/\sigma$ or m) was held constant throughout the computations.

A2.0 BINARY PROBIT MODEL

A2.1 MLE Algorithm for Binary Probit Model.

For each trial condition, i, there is a likelihood, L_I, of the observed result occurring:

$$L_i = p_i^{x_i} (1 - p_i)^{n_i - x_i} \qquad \text{(A1a)}$$

$$\log_e(L_i) = x_i [\log_e p_i] + (n_i - x_i) [\log_e (1 - p_i)] \quad \text{(A1b)}$$

where p_i is the event probability for test condition i, n_i is the number of independent trials under the i-th condition, and x_i is the number of successes in n_i. The likelihoods for all test conditions are then multiplied together to arrive at the likelihood, L. The values of the p_i's that are most supported by the quantal data are the values for which L is the largest. For ease of calculation, the natural logarithm of the likelihood is often used.

For a normal distribution, p_i is defined by the following relations:

$$p_i = \int_{-\infty}^{Z_i} f(Z)dZ \quad \text{(A2)}$$

$$f(Z) = \left[\frac{1}{\sqrt{2\pi}}\right] \exp\left[\frac{-Z^2}{2}\right] \quad \text{(A3)}$$

where Z, is the standard normal random variable and $f(Z)$ is the probability density function (pdf) of a standard normal distribution. In toxicology, the values of the individual p_i's are a function of the applied dosages, $(CT)_i$, used in an experiment and their respective distances from the median effective dosage, ECT_{50}. This is reflected in the following definition of Z_i:

$$Z_i = \frac{\{s_i - \mu\}}{\sigma} = m\{\log_{10}(CT)_i - \log_{10}(ECT_{50})\} \quad \text{(A4)}$$

where s_i is the applied stress for trial condition i and m is the probit slope (equal to $1/\sigma$). The 50% response level (or $p = 0.5$) corresponds to a Z value of zero. The MLE estimate of $\log(ECT_{50})$ is the value of $\log(ECT_{50})$ that is found to maximize L in eq A1.

For the MLE calculations, the first and second derivatives of $\log_e(L_i)$ with respect to μ are used:[3,7,8]

$$\left(\frac{\partial \log_e(L_i)}{\partial \mu}\right) = \left(\frac{f(Z_i)}{\sigma}\right)\left\{(-x_i)\left[\frac{1}{p_i}\right] + (n_i - x_i)\left[\frac{1}{(1-p_i)}\right]\right\} \quad \text{(A6)}$$

$$\left(\frac{\partial^2 \log_e(L_i)}{\partial \mu^2}\right) = \left(\frac{f(Z_i)}{\sigma^2}\right)\left\{(-x_i)\left[\frac{Z_i}{p_i} + \frac{f(Z_i)}{p_i^2}\right] + (n_i - x_i)\left[\frac{Z_i}{(1-p_i)} - \frac{f(Z_i)}{(1-p_i)^2}\right]\right\} \quad \text{(A7)}$$

To reach convergence at the value of $\log(ECT_{50})$ that maximizes $\log_e L$, a Newton-Raphson (or Newton's Method) algorithm (or similar procedure) can be used.[3,7,8,10] Using the Newton-Raphson method for the present system of equations (eqs A1b, A6, and A7), the

following equation is used to determine the next guess for log(ECT_{50}), as well as to check on convergence at the MLE for log(ECT_{50}):

$$\mu_{next} = \mu_o - \left(\frac{\partial \log_e L}{\partial \mu_o}\right) \Big/ \left(\frac{\partial^2 \log_e L}{\partial \mu_o^2}\right) \tag{A8}$$

where μ_o is the current guess for μ (or log(ECT_{50})), μ_{next} is the next guess for μ, and

$$\left(\frac{\partial \log_e L}{\partial \mu}\right) = \sum_{i=1}\left(\frac{\partial \log_e (L_i)}{\partial \mu}\right) \tag{A9}$$

$$\left(\frac{\partial^2 \log_e L}{\partial \mu^2}\right) = \sum_{i=1}\left(\frac{\partial^2 \log_e (L_i)}{\partial \mu^2}\right) \tag{A10}$$

The first and second derivatives for $\log_e L$ are evaluated at μ_o. $\text{Log}_e L$ is maximized when its first derivative with respect to μ equals zero. Convergence is achieved when the absolute difference between μ_o and μ_{next} is less than a predetermined value.

Thus, the following algorithm is used to find the MLE estimate for ECT_{50}:

(1) Set the probit slope (m) equal to some fixed value for the duration of the algorithm.

(2) Make an initial guess, μ_o, for μ [or log(ECT_{50})].

(3) Calculate Z_i, $f(Z_i)$ and p_i for each test condition i, corresponding to some $(CT)_i$ exposure using eqs A2, A3, and A4.

(4) Using eq A1, calculate the individual likelihoods, L_i.

(5) Multiply the L_i's (or add the $\log_e(L_i)$'s) together to estimate the total likelihood, L (or $\log_e L$), of the MLE estimate.

(6) Calculate the first and second derivatives for $\log_e L$ (evaluated at μ_o) using eqs A6, A7, A9, and A10.

(7) Check to verify whether the maximum value of L has been obtained. If not, go back to Step (3) with a new guess, μ_{next}, for μ [or log(ECT_{50})] using eq A8.

After the final log(ECT_{50}) estimate, $\hat{\mu}$, is obtained, there are three common and general methods for obtaining approximate confidence limits for the estimate:[3] Wald test, likelihood-ratio test, and the score (or Lagrange-multiplier) test. These approximations grow more accurate as the sample size gets larger.

In the present study, the Wald test was used to calculate confidence limits. Limits from the Wald test can be readily obtained from calculations performed as part of the Newton-Raphson algorithm used for finding the maximum value for L. However, the likelihood-ratio test required additional Newton-Raphson algorithm iterations.

In the present study, the following equation was used (based on the Wald test) to calculate the 95% asymptotic confidence interval for μ or the $\log(ECT_{50})$:[3]

$$\hat{\mu} - \frac{(1.96)}{\sqrt{\frac{-d^2 \log_e L(\hat{\mu})}{d\mu^2}}} \leq \mu \leq \hat{\mu} + \frac{(1.96)}{\sqrt{\frac{-d^2 \log_e L(\hat{\mu})}{d\mu^2}}} \qquad \text{(A11)}$$

where the second derivatives for $\log_e L$ are evaluated at $\hat{\mu}$ using eqs A7 and A10.

A2.2 Example of Application of Binary Probit Model with Fixed Probit Slope.

The following are the binary data for the 10-min exposures of the male pig to GB vapor. Dosage is in units of mg-min/m^3.

Table A1: Male Pig GB Ordinal Data (10-min exposure-duration)

Pig	Dosage (CT)	$\log_{10}$(CT)	Outcome	x_i
1	53.5	1.728354	< severe	0
2	59.0	1.770852	severe	0
3	67.0	1.826075	death	1
4	74.5	1.872156	severe	0
5	94.0	1.973128	death	1
6	95.0	1.977724	death	1

For this example, test condition i will only have one pig. So, for eq A1, n will equal one for each test condition. The values for x_i correspond to the absence or presence of lethality in the exposed pig.

Steps (1) and (2): Probit Slope and Initial Guess for $\log_{10}(ECT_{50})$ for Iteration One

For Step (1) of the algorithm, the probit slope is set equal to 10, which was used as the step size for the up and down method employed in the present study. For Step (2), the initial guess for the $\log_{10}(ECT_{50})$ is 1.85304.

Step (3): Calculation of Z_i's and p_i for Iteration One Using eqs A2, A3, and A4

$Z_1 = [1.72835) - 1.85304)] / (1/10) = -1.24685$

$\Longrightarrow p_1 = 0.10623$

$Z_2 = [1.77085) - 1.85304)] / (1/10) = -0.82187$

$\Longrightarrow p_2 = 0.20558$

$Z_3 = [1.82608) - 1.85304)] / (1/10) = -0.26964$

$\Longrightarrow p_3 = 0.39372$

$Z_4 = [1.87216) - 1.85304)] / (1/10) = 0.19117$

$\Longrightarrow p_4 = 0.57580$

$Z_5 = [1.97313) - 1.85304)] / (1/10) = 1.20089$

$\Longrightarrow p_5 = 0.88510$

$Z_6 = [1.97772) - 1.85304)] / (1/10) = 1.24685$

$\Longrightarrow p_6 = 0.89377$

Steps (4) and (5): Calculation of L_i's and L for Iteration One Using eq A1

$\log_e(L_1) = (0)(\log_e(0.10623)) + (1 - 0)(\log_e(1 - 0.10623)) = -0.11230$

$\log_e(L_2) = (0)(\log_e(0.20558)) + (1 - 0)(\log_e(1 - 0.20558)) = -0.23014$

$\log_e(L_3) = (1)(\log_e(0.39372)) + (1 - 1)(\log_e(1 - 0.39372)) = -0.93212$

$\log_e(L_4) = (0)(\log_e(0.57580)) + (1 - 0)(\log_e(1 - 0.57580)) = -0.85756$

$\log_e(L_5) = (1)(\log_e(0.88510)) + (1 - 1)(\log_e(1 - 0.88510)) = -0.12205$

$\log_e(L_6) = (1)(\log_e(0.89377)) + (1 - 1)(\log_e(1 - 0.89377)) = -0.11230$

Sum of the above $\log_e(L_i)$'s (or $\log_e(L)$) equals -2.36647.

Step (6): Calculate the First and Second Derivatives for $\log_e L$ (evaluated at μ_o) Using eqs A6, A7, A9 and A10

$d[\log_e(L_1)]/d[\mu_o] = 2.05162$

$d[\log_e(L_2)]/d[\mu_o] = 3.58246$

$d[\log_e(L_3)]/d[\mu_o] = -9.77094$

$d[\log_e(L_4)]/d[\mu_o] = 9.23437$

$d[\log_e(L_5)]/d[\mu_o] = -2.19159$

$d[\log_e(L_6)]/d[\mu_o] = -2.05162$

Sum of the above $d[\log_e(L_1)]/d[\mu_o]$'s equals 0.85430.

$d^2[\log_e(L_1)]/d[\mu_o]^2 = -29.790$

$d^2[\log_e(L_2)]/d[\mu_o]^2 = -42.277$

$d^2[\log_e(L_3)]/d[\mu_o]^2 = -69.125$

$d^2[\log_e(L_4)]/d[\mu_o]^2 = -67.620$

$d^2[\log_e(L_5)]/d[\mu_o]^2 = -31.122$

$d^2[\log_e(L_6)]/d[\mu_o]^2 = -29.790$

Sum of the above $d^2[\log_e(L_1)]/d[\mu_o]^2$'s equals -269.72.

Step (7): Check for Convergence on Maximum L Value and New Guess for $\log(ECT_{50})$ for Iteration Two Using eq A8

After the first iteration, the next guess for μ is found to equal:

$\mu_{next} = 1.85304 - (0.85430) / (-269.72) = 1.85304 - (-0.00317) = 1.85621$

Convergence was nearly reached after the first iteration, as seen above with only a difference of –0.00317 between μ_{next} and μ_o. After the second iteration, the difference falls further to 2.1×10^{-7}. Thus, the final estimate for $\log_{10}(ECT_{50})$ is 1.85621 (or $(ECT_{50}) = 71.8$ mg-min/m^3), and the final $\log_e(L)$ value was –2.36512. The denominators of eq A11 were found to equal the square root of 269.7. With this value for the denominators, the corresponding 95% asymptotic confidence interval for $\log_{10}(ECT_{50})$ equals 1.73686 to 1.97556, or for ECT_{50}, the interval is 54.6 to 94.5 mg-min/m^3.

A3.0 ORDINAL PROBIT MODEL

A3.1 MLE Algorithm for Ordinal Probit Model

To model an ordinal ternary response, eq A1 is modified as follows for each trial condition *i*:

$$L_i = p_{1,i}^{x_{1,i}} \, p_{2,i}^{x_{2,i}} (1 - p_{1,i} - p_{2,i})^{n_i - x_{1,i} - x_{2,i}} \tag{A12a}$$

$$\log_e(L_i) = x_{1,i} [\log_e p_{1,i}] + x_{2,i} [\log_e p_{2,i}] + (n_i - x_{1,i} - x_{2,i}) [\log_e (1 - p_{1,i} - p_{2,i})] \tag{A12b}$$

where $p_{1,i}$ and $p_{2,i}$ are the event probabilities for responses of categories one and two, respectively, for test condition *i*, n_i is the number of independent trials under the *i*-th condition, and $x_{1,i}$ and $x_{2,i}$ are the number of responses of categories one and two, respectively, in n_i. As before with the binary model, the likelihood, *L*, is obtained by multiplying together the individual likelihoods (for all test conditions).

The ordinal categories are in order of increasing severity, with category two being more severe than category one. For this example, category two corresponds to lethality, while category one corresponds to severe effects. Two normal distributions are represented in eq 12 and are defined as follows for the *i*-th condition:

$$p_{2,i} = \int_{-\infty}^{Z_{2,i}} f(Z) dZ \tag{A13}$$

$$p_{1,i} + p_{2,i} = \int_{-\infty}^{Z_{1,i}} f(Z) dZ \tag{A14}$$

The values of the individual $p_{1,i}$'s and $p_{2,i}$'s are a function of the applied dosages, $(CT)_i$, used in an experiment and their respective distances from their corresponding median effective dosages: for category one, μ_1 or ECT_{50} (severe); and for category two, μ_2 or LCT_{50} (lethality). This is reflected in the following definitions of the Z_i's for each *i*-th condition:

$$Z_{1,i} = \frac{\{s_i - \mu_1\}}{\sigma} = m \{\log_{10}(CT)_i - \log_{10}(ECT_{50})\} \tag{A15}$$

$$Z_{2,i} = \frac{\{s_i - \mu_2\}}{\sigma} = m \{\log_{10}(CT)_i - \log_{10}(LCT_{50})\} \tag{A16}$$

where $\mu_2 > \mu_1$ and σ (or *m*) is assumed to be the same for both distributions. MLE is now used to simultaneously obtain estimates for both the ECT_{50} (severe) and LCT_{50}. However, some

modifications are needed to eqs A6 and A7 to account for the first and second derivatives of $\log_e(L_i)$ with respect to μ_1 and μ_2, respectively:

$$\left(\frac{\partial \log_e(L_i)}{\partial \mu_1}\right) = \left(\frac{f(Z_{1,i})}{\sigma}\right)\left\{(-x_{1,i})\left[\frac{1}{p_{1,i}}\right] + (n_i - x_{1,i} - x_{2,i})\left[\frac{1}{p_{0,i}}\right]\right\} \tag{A17}$$

$$\left(\frac{\partial^2 \log_e(L_i)}{\partial \mu_1^2}\right) = \left(\frac{f(Z_{1,i})}{\sigma^2}\right)\left\{(-x_{1,i})\left[\frac{Z_{1,i}}{p_{1,i}} + \frac{f(Z_{1,i})}{p_{1,i}^2}\right] + (n_i - x_{1,i} - x_{2,i})\left[\frac{Z_{1,i}}{p_{0,i}} - \frac{f(Z_{1,i})}{p_{0,i}^2}\right]\right\} \tag{A18}$$

$$\left(\frac{\partial \log_e(L_i)}{\partial \mu_2}\right) = \left(\frac{f(Z_{2,i})}{\sigma}\right)\left\{(x_{1,i})\left[\frac{1}{p_{1,i}}\right] + (-x_{2,i})\left[\frac{1}{p_{2,i}}\right]\right\} \tag{A19}$$

$$\left(\frac{\partial^2 \log_e(L_i)}{\partial \mu_2^2}\right) = \left(\frac{f(Z_{2,i})}{\sigma^2}\right)\left\{(x_{1,i})\left[\frac{Z_{2,i}}{p_{1,i}} - \frac{f(Z_{2,i})}{p_{1,i}^2}\right] + (-x_{2,i})\left[\frac{Z_{2,i}}{p_{2,i}} + \frac{f(Z_{2,i})}{p_{2,i}^2}\right]\right\} \tag{A20}$$

where $p_{0,i}$ equals $(1 - p_{1,i} - p_{2,i})$. Also, the partial derivative of $\log_e(L_i)$ with respect to both μ_1 and μ_2, is required:

$$\left(\frac{\partial^2 \log_e(L_i)}{\partial \mu_1 \partial \mu_2}\right) = \left(\frac{f(Z_{1,i}) f(Z_{2,i})}{\sigma^2}\right)\left[\frac{x_{1,i}}{p_{1,i}^2}\right] \tag{A21}$$

To reach convergence at the values for μ_1 (or ECT_{50} (severe)) and μ_2 (or LCT_{50} (lethality)) that maximizes $\log_e L$, a Newton-Raphson (or Newton's Method) algorithm (or similar procedure) can be used.[3,7,8,10] Using the Newton-Raphson method for the present system of equations (eqs A17 to A21), the following simultaneous equations are used to determine the next guess for the median effective dosages, as well as to check on the convergence of the solution:

$$-\left(\frac{\partial \log_e(L)}{\partial \mu_1}\right) = \left(\frac{\partial^2 \log_e(L)}{\partial \mu_1^2}\right)\Delta\mu_1 + \left(\frac{\partial^2 \log_e(L)}{\partial \mu_1 \partial \mu_2}\right)\Delta\mu_2 \tag{A22}$$

$$-\left(\frac{\partial \log_e(L)}{\partial \mu_2}\right) = \left(\frac{\partial^2 \log_e(L)}{\partial \mu_1 \partial \mu_2}\right)\Delta\mu_1 + \left(\frac{\partial^2 \log_e(L)}{\partial \mu_2^2}\right)\Delta\mu_2 \tag{A23}$$

where $\Delta\mu_1 = (\mu_{next} - \mu_o)_1$ and $\Delta\mu_2 = (\mu_{next} - \mu_o)_2$ for median effective dosages 1 (severe) and 2 (lethal), respectively, and

$$\left(\frac{\partial \log_e L}{\partial \mu_j}\right) = \sum_{i=1}\left(\frac{\partial \log_e (L_i)}{\partial \mu_j}\right) \tag{A24}$$

$$\left(\frac{\partial^2 \log_e L}{\partial \mu_j^2}\right) = \sum_{i=1}\left(\frac{\partial^2 \log_e (L_i)}{\partial \mu_j^2}\right) \tag{A25}$$

$$\left(\frac{\partial^2 \log_e L}{\partial \mu_1 \partial \mu_2}\right) = \sum_{i=1}\left(\frac{\partial \log_e (L_i)}{\partial \mu_1 \partial \mu_2}\right) \tag{A26}$$

where, j equals 1 and 2 for severe and lethality, respectively. The above derivatives for $\log_e L$ are evaluated at $\mu_{1,o}$ and $\mu_{2,o}$. $\text{Log}_e L$ is maximized when its first derivatives with respect to the two μ's equal zero. Convergence is achieved when the absolute difference between μ_o and μ_{next} is less than a predetermined value. After convergence is reached, the asymptotic variance-covariance matrix of the maximum likelihood estimate can be calculated by taking the inverse of the matrix of second derivatives of $\text{Log}_e L$:[3,7,8]

$$V(\mu_1, \mu_2) = \begin{bmatrix} \left(\frac{\partial^2 \log_e (L)}{\partial \mu_1^2}\right) & \left(\frac{\partial^2 \log_e (L)}{\partial \mu_1 \partial \mu_2}\right) \\ \left(\frac{\partial^2 \log_e (L)}{\partial \mu_1 \partial \mu_2}\right) & \left(\frac{\partial^2 \log_e (L)}{\partial \mu_2^2}\right) \end{bmatrix}^{-1} \tag{A27}$$

Thus, the following algorithm is used to find the MLE estimate for ECT_{50}:

(1) Set the probit slope (m) equal to some fixed value for the duration of the algorithm.

(2) Make initial guesses for $\log(ECT_{50})$ and $\log(LCT_{50})$: $\mu_{1,o}$ and $\mu_{2,o}$, respectively.

(3) Calculate $Z_{i,j}$, $f(Z_{i,j})$ and $p_{i,j}$ for each test condition i and mean dosage j, corresponding to some $(CT)_i$ using eqs A3 and A13 to A16.

(4) Using eq A12, calculate the individual likelihoods, L_i.

(5) Multiple the L_i's [or add the $\log_e(L_i)$'s] together to estimate the total likelihood, L (or $\log_e L$), of the MLE estimate.

(6) Calculate the first and second derivatives for $\log_e L$ (evaluated at μ_o) for each mean dosage j, using eqs A17 to A20. Also, calculate the derivative for $\log_e L$ with respect to both $\mu_{1,o}$ and $\mu_{2,o}$ using eq A21.

(7) Check to verify whether the maximum value of L has been obtained. If not, go back to Step (3) with new guesses, $\mu_{1,next}$ and $\mu_{2,next}$, for μ_1 and μ_2, respectively, by solving eqs A22 and A23, simultaneously.

In some instances with Step (7), poor initial guesses for μ_1 and μ_2 may produce the situation where $\mu_{1,next} > \mu_{2,next}$, a violation of a key boundary condition. A simple resolution for this problem is to artificially reduce (for this iteration) the values of $\Delta\mu_1$ and $\Delta\mu_2$ by some set factor.

After the final $\log(ECT_{50})$ and $\log(LCT_{50})$ estimates, $\hat{\mu}_1$ and $\hat{\mu}_2$, are obtained, there are three common and general methods for obtaining approximate confidence limits for these estimates:[3] Wald test, likelihood-ratio test, and the score (or Lagrange-multiplier) test. As with the binary probit model (see Section A2.0), these approximations grow more accurate as the sample size gets larger.

In the present study, the Wald test was used to calculate confidence limits. Limits from the Wald test can be readily obtained from calculations performed as part of the Newton-Raphson algorithm used for finding the maximum value for L. However, the likelihood-ratio test required additional Newton-Raphson algorithm iterations.

In the present study, the following equation was used (based on the Wald test) to calculate the 95% asymptotic confidence interval for each μ_j (with j = 1 for $\log(ECT_{50})$ and 2 for $\log(LCT_{50})$):[3,7,8]

$$\hat{\mu}_j - \frac{(1.96)}{\sqrt{\text{var}(\mu_j)}} \leq \mu_j \leq \hat{\mu}_j + \frac{(1.96)}{\sqrt{\text{var}(\mu_j)}} \qquad \text{(A28)}$$

where $\text{var}(\mu_j)$ is the variance for μ_j. The values for the variances and covariance are calculated using eq A27.

A3.2 Example of Application of Ordinal Probit Model with Fixed Probit Slope

The following are the quantal data for the ten-minute exposures of the male pig to GB vapor. Dosage is in units of mg-min/m^3.

Table A2: Male Pig GB Ordinal Data (10-min exposure-duration)

Pig	Dosage (CT)	$\log_{10}$(CT)	Outcome	x_1	x_2
1	53.5	1.728354	< severe	0	0
2	59.0	1.770852	severe	1	0
3	67.0	1.826075	death	0	1
4	74.5	1.872156	severe	1	0
5	94.0	1.973128	death	0	1
6	95.0	1.977724	death	0	1

For this example, test condition *i* will only have one pig. So, *n* will equal one for each test condition for eq A12. The values for x_1 and x_2 correspond to the absence or presence of a maximum effect: x_1 equals one if the maximum effect observed was severe effects (and equals zero otherwise); and x_2 equals one if the maximum effect was lethality (and equals zero otherwise).

Steps (1) and (2): Probit Slope and Initial Guesses for $\log_{10}(ECT_{50})$ and $\log_{10}(LCT_{50})$ for Iteration One

For Step (1) of the algorithm, the probit slope is set equal to 10, which was used as the step size for the up and down method employed in the present study. For Step (2), the initial guesses for $\log_{10}(ECT_{50})$ and $\log_{10}(LCT_{50})$ are 1.68 and 1.83, respectively.

Steps (3), (4) and (5): Calculation of $Z_{i,j}$, $f(Z_{i,j})$ and $p_{i,j}$ Using eqs A3 and A13 to A16 and of L_i's and L Using eq A12 for Iteration One

Table A3 shows the calculated values for each pig. The sum of the individual $\log_e L_i$ equals -3.8212.

Table A3: Values of $Z_{i,j}$, $f(Z_{i,j})$ and $p_{i,j}$ for First Iteration

Pig	Z_1	Z_2	$f(Z_1)$	$f(Z_2)$	p_1	p_2	$\text{Log}_e L_i$
1	0.48354	-1.01646	0.35493	0.23799	0.53094	0.15470	-1.15720
2	0.90852	-0.59148	0.26404	0.33492	0.54110	0.27710	-0.61420
3	1.46075	-0.03925	0.13727	0.39864	0.44361	0.48434	-0.72500
4	1.92156	0.42156	0.06297	0.36502	0.30934	0.66333	-1.17330
5	2.93128	1.43128	0.00543	0.14324	0.07449	0.92382	-0.07920
6	2.97724	1.47724	0.00474	0.13398	0.06835	0.93019	-0.07240

Step (6): Calculate the Various Derivatives for $\log_e L$ (evaluated at $\mu_{1,o}$ and $\mu_{2,o}$) for Each Mean Dosage *j*, Using eqs A17 to A21.

Table A4 shows the calculated values for each pig. Pigs showing severe effects make contributions to all five derivatives, whereas those pigs having less than severe effects or died only contribute to two of the five derivatives.

Table A4: Values of $\log_e L$ Derivatives for First Iteration

Pig	$\left(\frac{\partial \log_e(L_i)}{\partial \mu_1}\right)$	$\left(\frac{\partial^2 \log_e(L_i)}{\partial \mu_1^2}\right)$	$\left(\frac{\partial \log_e(L_i)}{\partial \mu_2}\right)$	$\left(\frac{\partial^2 \log_e(L_i)}{\partial \mu_2^2}\right)$	$\left(\frac{\partial^2 \log_e(L_i)}{\partial \mu_1 \partial \mu_2}\right)$
1	11.2906	-72.8829	0	0	0
2	-4.8798	-68.1456	6.1896	-74.9220	30.2039
3	0	0	-8.2304	-64.5089	0
4	-2.0355	-43.2572	11.8000	-89.4952	24.0191
5	0	0	-1.5505	-24.5966	0
6	0	0	-1.4404	-23.3522	0
Total	4.3753	-184.2857	6.7683	-276.8749	54.2231

Step (7): Check for Convergence on Maximum *L* Value and New Guesses for $\log(ECT_{50})$ and $\log(LCT_{50})$ for Iteration Two Using eqs A22 and A23.

Solving eqs A22 and A23 simultaneously produces values of 0.03283 and 0.03087 for $\Delta\mu_1$ and $\Delta\mu_2$, respectively. So, the next guesses for μ_1 and μ_2 are 1.71283 and 1.86087, respectively. After the third iteration, the absolute values for both $\Delta\mu_1$ and $\Delta\mu_2$ are less than 1×10^{-5}. At which point, the final values for μ_1 and μ_2 are 1.71203 and 1.86015, respectively. Thus, the final estimates for ECT_{50} and LCT_{50} are 51.5 and 72.5 mg-min/m^3, respectively. The final $\log_e(L)$ value was -3.6478. The final variance-covariance matrix equals:

$$V(\mu_1, \mu_2) = \begin{bmatrix} 0.005449 & 0.001100 \\ 0.001100 & 0.003642 \end{bmatrix}$$

Using the above information with eq A28, the corresponding 95% asymptotic confidence interval for $\log_{10}(ECT_{50})$ equals 1.56735 to 1.85671, or for ECT_{50}, the interval is 36.9 to 71.9 mg-min/m^3. For lethality, the confidence interval for $\log_{10}(LCT_{50})$ equals 1.74186 to 1.97845, or for LCT_{50}, the interval is 55.2 to 95.2 mg-min/m^3.

A4.0 OTHER USEFUL RELATIONSHIPS

For simplicity, many of the derivative equations are presented without benefit of showing intermediate steps. The following is a listing of relationships that were useful in arriving at the final relations.

A4.1 Binary Probit Model

$$\frac{\partial p_i}{\partial \mu} = \frac{-f(Z_i)}{\sigma} \tag{A29}$$

$$\frac{\partial f(Z)}{\partial \mu} = \left[\frac{f(Z)Z}{\sigma}\right] \tag{A30}$$

$$\frac{\partial Z_i}{\partial \mu} = -\left(\frac{\mathbf{1}}{\sigma}\right) \tag{A31}$$

A4.2 Ordinal Probit Model

$$\frac{\partial p_{1,i}}{\partial \mu_1} = \frac{-f(Z_{1,i})}{\sigma} \tag{A32}$$

$$\frac{\partial p_{2,i}}{\partial \mu_1} = 0 \tag{A33}$$

$$\frac{\partial p_{1,i}}{\partial \mu_2} = -\left(\frac{\partial p_{2,i}}{\partial \mu_2}\right) = \frac{f(Z_{2,i})}{\sigma} \tag{A34}$$

$$\frac{\partial p_{2,i}}{\partial \mu_2} = \frac{-f(Z_{2,i})}{\sigma} \tag{A35}$$

$$\frac{\partial f(Z_1)}{\partial \mu_1} = \left[\frac{f(Z_1)Z_1}{\sigma}\right] \tag{A36}$$

$$\frac{\partial f(Z_2)}{\partial \mu_2} = \left[\frac{f(Z_2)Z_2}{\sigma}\right] \tag{A37}$$

$$\frac{\partial f(Z_2)}{\partial \mu_1} = \frac{\partial f(Z_1)}{\partial \mu_2} = 0 \tag{A38}$$

$$\frac{\partial Z_1}{\partial \mu_1} = \frac{\partial Z_2}{\partial \mu_2} = -\left(\frac{\mathbf{1}}{\sigma}\right) \tag{A39}$$

$$\frac{\partial Z_1}{\partial \mu_2} = \frac{\partial Z_2}{\partial \mu_1} = \mathbf{0} \tag{A40}$$

A5.0 REFERENCES

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[4] **MINITAB™ Statistical Software**, Release 13.32. MINITAB Inc., 3081 Enterprise Drive, State College, PA 16801-3008, website: **/www.minitab.com/**. 2002.

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APPENDIX B

MINITAB PRINTOUTS (ALL PIGS)

Probit Analysis: Death versus CT, Group

```
Distribution:  Lognormal base 10

Response Information

Variable  Value       Count
Death     1              18  (Event)
          0              20
          Total          38

Factor Information

Factor   Levels Values
Group         6 M10  M60  M180 F10  F60  F180

Estimation Method:  Maximum Likelihood

Regression Table
                          Standard
Variable        Coef         Error         Z      P
Constant     -20.074         6.099     -3.29  0.001
CT            10.816         3.275      3.30  0.001
Group
 M60          -1.825         1.005     -1.82  0.069
 M180         -4.335         1.566     -2.77  0.006
 F10          -1.0542       0.9125     -1.16  0.248
 F60          -2.572         1.105     -2.33  0.020
 F180         -4.163         1.545     -2.69  0.007
Natural
Response       0.000

Test for equal slopes:  Chi-Square = 5.7971,  DF = 5,  P-Value = 0.326
Log-Likelihood = -17.891

Multiple degree of freedom test

Term       Chi-Square    DF      P
Group           8.823     5  0.116

Goodness-of-Fit Tests

Method                 Chi-Square    DF      P
Pearson                    39.051    30  0.125
Deviance                   35.783    30  0.215
Hosmer-Lemeshow             5.002     8  0.757

Table of Observed and Expected Frequencies:
(See Hosmer-Lemeshow Test for the Pearson Chi-Square Statistic)

                                  Group
Value       1     2     3     4     5     6     7     8     9    10   Total
1
  Obs       0     1     0     1     2     1     3     3     3     4      18
  Exp     0.0   0.4   0.9   1.2   1.6   1.5   3.2   2.2   3.5   3.8
0
```

Obs	3	3	4	3	2	2	2	0	1	0	20
Exp	3.0	3.6	3.1	2.8	2.4	1.5	1.8	0.8	0.5	0.2	
Total	3	4	4	4	4	3	5	3	4	4	38

Group = M10

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	71.7733	9.4814	52.2478	99.4268

Group = M60

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	105.8463	11.7477	79.7964	137.0774

Group = M180

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	180.6076	22.4920	133.8512	245.4973

Group = F10

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	89.8318	11.3579	66.5600	123.2342

Group = F60

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	124.0963	15.1873	94.6559	172.2615

Group = F180

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	174.1303	25.0783	123.9176	249.9727

Table of Relative Potency

Factor: Group

Comparison	Relative Potency	95.0% Fiducial CI Lower	Upper	
M10 VS M60	1.4747	0.9528	2.2100	
M10 VS M180	2.5164	1.6165	3.9131	
M10 VS F10	1.2516	0.8051	1.9612	not different
M10 VS F60	1.7290	1.1425	2.7473	

```
M10  VS F180      2.4261       1.5174       3.9296
M60  VS M180      1.7063       1.1541       2.6031
M60  VS F10       0.8487       0.5743       1.3056
M60  VS F60       1.1724       0.8136       1.8322 not different
M60  VS F180      1.6451       1.0796       2.6231
M180 VS F10       0.4974       0.3243       0.7696
M180 VS F60       0.6871       0.4603       1.0780
M180 VS F180      0.9641       0.6108       1.5434 not different
F10  VS F60       1.3814       0.9190       2.1630
F10  VS F180      1.9384       1.2190       3.0979
F60  VS F180      1.4032       0.8707       2.1819

MTB > code (1)0 (2 3)1 'Score' 'Severe'
MTB > Probit  'Severe' = 'CT' 'Group';
SUBC>   Factors 'Group';
SUBC>   Lgnten;
SUBC>   Brief 3;
SUBC>   Confidence 95.0.
```

Probit Analysis: Severe versus CT, Group

```
Distribution:  Lognormal base 10

Response Information

Variable  Value       Count
Severe    1              27  (Event)
          0              11
          Total          38

Factor Information

Factor   Levels Values
Group         6 M10  M60  M180 F10  F60  F180

Estimation Method:  Maximum Likelihood

Regression Table
                          Standard
Variable        Coef         Error         Z      P
Constant     -16.783         6.417     -2.62  0.009
CT             9.793         3.568      2.74  0.006
Group
 M60          -2.038         1.285     -1.59  0.113
 M180         -4.095         1.852     -2.21  0.027
 F10          -1.776         1.024     -1.73  0.083
 F60          -3.119         1.279     -2.44  0.015
 F180         -4.483         1.789     -2.51  0.012
Natural
Response       0.000

Test for equal slopes:  Chi-Square = 3.7545,  DF = 5,  P-Value = 0.585
Log-Likelihood = -15.195

Multiple degree of freedom test

Term       Chi-Square    DF      P
Group           7.790     5  0.168

Goodness-of-Fit Tests

Method                 Chi-Square    DF      P
```

Pearson	27.976	30	0.572
Deviance	30.389	30	0.446
Hosmer-Lemeshow	5.001	8	0.757

Table of Observed and Expected Frequencies:
(See Hosmer-Lemeshow Test for the Pearson Chi-Square Statistic)

	Group									
Value	1	2	3	4	5	6	7	8	9	10	Total
1											
Obs	0	2	2	2	2	3	4	4	4	4	27
Exp	0.2	1.4	2.1	2.7	3.2	2.6	3.5	3.7	3.9	4.0	
0											
Obs	3	2	2	2	2	0	0	0	0	0	11
Exp	2.8	2.6	1.9	1.3	0.8	0.4	0.5	0.3	0.1	0.0	
Total	3	4	4	4	4	3	4	4	4	4	38

Group = M10

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	51.7458	9.6069	25.1350	75.2237

Group = M60

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	83.5637	14.1420	38.7730	111.9375

Group = M180

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	135.5373	24.5361	63.7336	190.5009

Group = F10

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	78.5629	10.0979	52.7437	108.7298

Group = F60

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
50	107.7515	15.2617	73.9244	163.5522

Group = F180

Table of Percentiles

		Standard	95.0% Fiducial CI

```
 Percent  Percentile       Error       Lower       Upper
      50    148.4678     23.2337     88.1604    214.5667
```

```
Table of Relative Potency

Factor:  Group
                  Relative          95.0% Fiducial CI
Comparison         Potency       Lower          Upper
M10  VS M60         1.6149      0.7930         2.8947
M10  VS M180        2.6193      1.2872         4.9888
M10  VS F10         1.5182      0.9208         3.2940 not different
M10  VS F60         2.0823      1.2722         5.0262
M10  VS F180        2.8692      1.6220         6.1679
M60  VS M180        1.6220      0.8974         3.1172
M60  VS F10         0.9402      0.6218         2.1251
M60  VS F60         1.2895      0.8486         3.2828 not different
M60  VS F180        1.7767      1.1027         3.9527
M180 VS F10         0.5796      0.3649         1.2943
M180 VS F60         0.7950      0.5014         1.9863
M180 VS F180        1.0954      0.6452         2.4146 not different
F10  VS F60         1.3715      0.8463         2.4912
F10  VS F180        1.8898      1.0337         3.1909
F60  VS F180        1.3779      0.6856         2.2820
```

```
MTB > OLogistic 'Score' = logC Time Gender Time*Gender;
SUBC>   Factors 'Time' 'Gender';
SUBC>   Normit;
SUBC>   Brief 3.
```

Model L1

Ordinal Logistic Regression: Score versus logC, Time, Gender

```
Link Function:  Normit

Response Information

Variable  Value       Count
Score     1              11
          2               9
          3              18
          Total          38

Factor Information

Factor   Levels Values
Time          3  10  60 180
Gender        2 female male

Logistic Regression Table

Predictor          Coef    SE Coef       Z      P
Const(1)          9.344      2.574    3.63  0.000
Const(2)         10.268      2.646    3.88  0.000
logC            -10.698      2.765   -3.87  0.000
Time
  60             -6.624      1.891   -3.50  0.000
 180            -10.363      2.776   -3.73  0.000
Gender
 male            -1.3012     0.7854  -1.66  0.098
```

```
Time*Gender
  60*male          0.248       1.031      0.24 0.810
 180*male          1.287       1.104      1.17 0.243

Tests for terms with more than 1 degree of freedom

Term          Chi-Square    DF      P
Time              13.936     2  0.001
Time*Gender        1.547     2  0.461

Log-likelihood = -29.365
Test that all slopes are zero: G = 21.369, DF = 6, P-Value = 0.002

Goodness-of-Fit Tests

Method       Chi-Square    DF      P
Pearson          77.645    66  0.155
Deviance         58.731    66  0.725

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs           Number  Percent     Summary Measures
Concordant         390    85.0%     Somers' D                0.70
Discordant          67    14.6%     Goodman-Kruskal Gamma    0.71
Ties                 2     0.4%     Kendall's Tau-a          0.46
Total              459   100.0%

MTB > OLogistic 'Score' = logC Time Gender ;
SUBC>   Factors 'Time' 'Gender';
SUBC>   Normit;
SUBC>   Brief 3.
```

Model L2

Ordinal Logistic Regression: Score versus logC, Time, Gender

```
Link Function:  Normit

Response Information

Variable  Value       Count
Score     1              11
          2               9
          3              18
          Total          38

Factor Information

Factor   Levels Values
Time          3  10  60 180
Gender        2 female male

Logistic Regression Table

Predictor       Coef    SE Coef       Z      P
Const(1)       8.353      2.321    3.60 0.000
Const(2)       9.250      2.389    3.87 0.000
logC          -9.825      2.591   -3.79 0.000
Time
```

```
  60          -5.992       1.646     -3.64 0.000
 180          -8.904       2.394     -3.72 0.000
Gender
 male        -0.7947      0.4274     -1.86 0.063

Tests for terms with more than 1 degree of freedom

Term       Chi-Square    DF      P
Time           13.880     2  0.001

Log-likelihood = -30.139
Test that all slopes are zero: G = 19.821, DF = 4, P-Value = 0.001

Goodness-of-Fit Tests

Method       Chi-Square    DF      P
Pearson          71.098    68  0.375
Deviance         60.278    68  0.736

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs            Number  Percent     Summary Measures
Concordant          377    82.1%     Somers' D                0.64
Discordant           82    17.9%     Goodman-Kruskal Gamma    0.64
Ties                  0     0.0%     Kendall's Tau-a          0.42
Total               459   100.0%

MTB > OLogistic 'Score' = logC Time  ;
SUBC>   Factors 'Time' ;
SUBC>   Normit;
SUBC>   Brief 3.
```

Model L3

Ordinal Logistic Regression: Score versus logC, Time

```
Link Function:  Normit

Response Information

Variable  Value       Count
Score     1              11
          2               9
          3              18
          Total          38

Factor Information

Factor   Levels Values
Time          3  10  60 180

Logistic Regression Table

Predictor       Coef    SE Coef         Z      P
Const(1)       7.292      2.156      3.38 0.001
Const(2)       8.125      2.212      3.67 0.000
logC          -9.016      2.463     -3.66 0.000
Time
  60          -5.540      1.587     -3.49 0.000
 180          -8.220      2.278     -3.61 0.000
```

```
Tests for terms with more than 1 degree of freedom

Term      Chi-Square    DF      P
Time          13.020     2  0.001

Log-likelihood = -31.919
Test that all slopes are zero: G = 16.261, DF = 3, P-Value = 0.001

Goodness-of-Fit Tests

Method       Chi-Square    DF      P
Pearson          75.833    69  0.268
Deviance         63.839    69  0.653

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs            Number  Percent     Summary Measures
Concordant          361    78.6%     Somers' D                0.58
Discordant           97    21.1%     Goodman-Kruskal Gamma    0.58
Ties                  1     0.2%     Kendall's Tau-a          0.38
Total               459   100.0%

MTB > Name m2 = 'XPWX2'
MTB > OLogistic 'Score' = logC logT Sex  Sex*logT;
SUBC>   Normit;
SUBC>   XPWXinverse 'XPWX2';
SUBC>   Brief 3.
```

Model L4

Ordinal Logistic Regression: Score versus logC, logT, Sex

```
Link Function:  Normit

Response Information

Variable  Value       Count
Score     1              11
          2               9
          3              18
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef        Z      P
Const(1)      15.063      4.144     3.63  0.000
Const(2)      15.958      4.212     3.79  0.000
logC          -9.865      2.590    -3.81  0.000
logT          -7.207      1.924    -3.74  0.000
Sex           -1.1428     0.7731   -1.48  0.139
logT*Sex       0.4457     0.4342    1.03  0.305

Log-likelihood = -30.147
Test that all slopes are zero: G = 19.805, DF = 4, P-Value = 0.001

Goodness-of-Fit Tests

Method       Chi-Square    DF      P
Pearson          78.211    68  0.186
```

```
Deviance          60.294     68  0.736

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs            Number  Percent     Summary Measures
Concordant          382    83.2%     Somers' D                0.67
Discordant           76    16.6%     Goodman-Kruskal Gamma    0.67
Ties                  1     0.2%     Kendall's Tau-a          0.44
Total               459   100.0%

MTB > print m2
```

Data Display

```
 Matrix XPWX2

  17.1740   17.4202  -10.5623   -7.9504   -1.0833    0.5123
  17.4202   17.7383  -10.7412   -8.0818   -1.1036    0.5202
 -10.5623  -10.7412    6.7076    4.8670    0.7074   -0.3337
  -7.9504   -8.0818    4.8670    3.7035    0.4997   -0.2371
  -1.0833   -1.1036    0.7074    0.4997    0.5977   -0.3224
   0.5123    0.5202   -0.3337   -0.2371   -0.3224    0.1885

MTB > Name m3 = 'XPWX3'
MTB > OLogistic 'Score' = logC logT Sex  ;
SUBC>   Normit;
SUBC>   XPWXinverse 'XPWX3';
SUBC>   Brief 3.
```

Model L5

Ordinal Logistic Regression: Score versus logC, logT, Sex

```
Link Function:  Normit

Response Information

Variable  Value       Count
Score     1              11
          2               9
          3              18
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef       Z      P
Const(1)      14.004      3.926    3.57  0.000
Const(2)      14.877      3.990    3.73  0.000
logC          -9.181      2.448   -3.75  0.000
logT          -6.711      1.824   -3.68  0.000
Sex           -0.3901     0.2126  -1.83  0.067

Log-likelihood = -30.692
Test that all slopes are zero: G = 18.716, DF = 3, P-Value = 0.000

Goodness-of-Fit Tests

Method       Chi-Square    DF      P
Pearson          79.671    69  0.178
```

```
Deviance        61.384    69  0.731

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs            Number  Percent     Summary Measures
Concordant          374    81.5%     Somers' D                0.63
Discordant           84    18.3%     Goodman-Kruskal Gamma    0.63
Ties                  1     0.2%     Kendall's Tau-a          0.41
Total               459   100.0%

MTB > print m3
```

Data Display

```
 Matrix XPWX3

  15.4117   15.6317   -9.4455   -7.1341   -0.1913
  15.6317   15.9208   -9.6069   -7.2526   -0.1981
  -9.4455   -9.6069    5.9921    4.3492    0.1239
  -7.1341   -7.2526    4.3492    3.3254    0.0879
  -0.1913   -0.1981    0.1239    0.0879    0.0452

MTB > Name m4 = 'XPWX4'
MTB > OLogistic 'Score' = logC logT ;
SUBC>   Normit;
SUBC>   XPWXinverse 'XPWX4';
SUBC>   Brief 3.
```

Model L6

Ordinal Logistic Regression: Score versus logC, logT

```
Link Function:  Normit

Response Information

Variable  Value       Count
Score     1              11
          2               9
          3              18
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef       Z      P
Const(1)      12.838      3.690    3.48  0.001
Const(2)      13.651      3.747    3.64  0.000
logC          -8.402      2.303   -3.65  0.000
logT          -6.180      1.719   -3.59  0.000

Log-likelihood = -32.428
Test that all slopes are zero: G = 15.244, DF = 2, P-Value = 0.000

Goodness-of-Fit Tests

Method       Chi-Square    DF      P
Pearson          78.460    70  0.228
Deviance         64.856    70  0.651
```

```
Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs            Number  Percent     Summary Measures
Concordant          356    77.6%     Somers' D                0.56
Discordant          101    22.0%     Goodman-Kruskal Gamma    0.56
Ties                  2     0.4%     Kendall's Tau-a          0.36
Total               459   100.0%

MTB > print m4
```

Data Display

```
Matrix XPWX4

  13.6191   13.7995   -8.3393   -6.3225
  13.7995   14.0401   -8.4723   -6.4208
  -8.3393   -8.4723    5.3030    3.8495
  -6.3225   -6.4208    3.8495    2.9563
```

Ordinal Logistic Regression: Score versus logC, logT, Sex, LogT45sq

```
Link Function:  Normit

Response Information

Variable  Value       Count
Score     1              11
          2               9
          3              18
          Total          38

Logistic Regression Table

Predictor       Coef    SE Coef       Z      P
Const(1)      14.442      4.039    3.58  0.000
Const(2)      15.339      4.106    3.74  0.000
logC          -9.825      2.591   -3.79  0.000
logT          -7.028      1.894   -3.71  0.000
Sex           -0.3973     0.2137  -1.86  0.063
LogT45sq       1.272      1.223    1.04  0.299  no curvature;
          The last term is [Log(T/45)]^2.

Log-likelihood = -30.139
Test that all slopes are zero: G = 19.821, DF = 4, P-Value = 0.001

Goodness-of-Fit Tests

Method       Chi-Square    DF      P
Pearson          71.098    68  0.375
Deviance         60.278    68  0.736

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs            Number  Percent     Summary Measures
Concordant          377    82.1%     Somers' D                0.64
Discordant           82    17.9%     Goodman-Kruskal Gamma    0.64
Ties                  0     0.0%     Kendall's Tau-a          0.42
Total               459   100.0%

MTB > print m5
```

Data Display

```
Matrix XPWX1

  16.3159   16.5512  -10.2044   -7.6127   -0.1809    0.6725
  16.5512   16.8588  -10.3830   -7.7411   -0.1885    0.6955
 -10.2044  -10.3830    6.7116    4.7776    0.1195   -0.8497
  -7.6127   -7.7411    4.7776    3.5871    0.0832   -0.4464
  -0.1809   -0.1885    0.1195    0.0832    0.0457   -0.0039
   0.6725    0.6955   -0.8497   -0.4464   -0.0039    1.4964
```

MINITAB PRINTOUTS (WITHOUT PIG 63)

Model L1

Ordinal Logistic Regression: Score versus logC, Time, sex

```
Link Function: Normit

Response Information

Variable  Value  Count
Score     0         11
          1          9
          2         17
          Total     37

Factor Information

Factor  Levels  Values
Time         3  10, 60, 180
sex          2  female, male

* NOTE * 37 cases were used
* NOTE * 1 cases contained missing values

[DRS] Female Pig 63 is an outlier and was dropped from this analysis.

Logistic Regression Table

Predictor       Coef   SE Coef      Z      P
Const(1)     13.8594   3.66928   3.78  0.000
Const(2)     15.0153   3.78178   3.97  0.000
logC        -15.7332   3.98037  -3.95  0.000
Time
  60        -8.64404   2.34381  -3.69  0.000
 180        -15.1925   3.89458  -3.90  0.000
sex
 male       -1.81609  0.922677  -1.97  0.049
Time*sex
  60*male  -0.632034   1.13371  -0.56  0.577
 180*male    1.88576   1.22680   1.54  0.124

Tests for terms with more than 1 degree of freedom

Term      Chi-Square  DF      P
Time         15.2175   2  0.000
```

```
Time*sex      4.2498   2  0.119

Log-Likelihood = -24.252
Test that all slopes are zero: G = 30.070, DF = 6, P-Value = 0.000

Goodness-of-Fit Tests

Method     Chi-Square  DF      P
Pearson       61.0914  64  0.580
Deviance      48.5048  64  0.925

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs        Number  Percent  Summary Measures
Concordant      390     88.8  Somers' D              0.78
Discordant       48     10.9  Goodman-Kruskal Gamma  0.78
Ties              1      0.2  Kendall's Tau-a        0.51
Total           439    100.0

MTB > print m2
```

Data Display

```
Matrix XPWX4

 13.4636   13.8243  -14.4182  -8.22759  -14.1090  -1.94651   0.04925   2.09104
 13.8243   14.3018  -14.8744  -8.48282  -14.5525  -1.99799   0.03072   2.14581
-14.4182  -14.8744   15.8434   8.69882   15.1628   1.79405   0.29641  -1.95214
 -8.2276   -8.4828    8.6988   5.49343    8.6392   1.29877  -0.55390  -1.38551
-14.1090  -14.5525   15.1628   8.63922   15.1677   2.03072  -0.02941  -2.52403
 -1.9465   -1.9980    1.7940   1.29877    2.0307   0.85133  -0.61463  -0.86924
  0.0492    0.0307    0.2964  -0.55390   -0.0294  -0.61463   1.28531   0.61180
  2.0910    2.1458   -1.9521  -1.38551   -2.5240  -0.86924   0.61180   1.50503
```

Model L2

Ordinal Logistic Regression: Score versus logC, Time, sex

```
Link Function: Normit

Response Information

Variable  Value  Count
Score     0         11
          1          9
          2         17
          Total     37

Factor Information

Factor  Levels  Values
Time         3  10, 60, 180
sex          2  female, male

* NOTE * 37 cases were used
* NOTE * 1 cases contained missing values

[DRS] Female Pig 63 is an outlier and was dropped from this analysis.
```

```
Logistic Regression Table

Predictor       Coef   SE Coef      Z      P
Const(1)     10.9275   2.87594   3.80  0.000
Const(2)     11.9844   2.97145   4.03  0.000
logC        -12.6430   3.19098  -3.96  0.000
Time
  60         -7.34827  1.92722  -3.81  0.000
 180         -11.4296  2.91644  -3.92  0.000
sex
 male        -1.19785  0.488518 -2.45  0.014
Tests for terms with more than 1 degree of freedom

Term  Chi-Square  DF      P
Time     15.3604   2  0.000

Log-Likelihood = -26.484
Test that all slopes are zero: G = 25.607, DF = 4, P-Value = 0.000

Goodness-of-Fit Tests

Method     Chi-Square  DF      P
Pearson       58.8779  66  0.721
Deviance      52.9675  66  0.877

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs        Number  Percent  Summary Measures
Concordant      379     86.3  Somers' D              0.73
Discordant       60     13.7  Goodman-Kruskal Gamma  0.73
Ties              0      0.0  Kendall's Tau-a        0.48
Total           439    100.0

MTB > print m3
```

Data Display

```
 Matrix XPWX5

 8.27103   8.50165  -9.0598  -5.39319  -8.27102  -0.675879
 8.50165   8.82949  -9.3671  -5.57286  -8.55020  -0.705401
-9.05980  -9.36708  10.1824   5.91670   9.13973   0.657970
-5.39319  -5.57286   5.9167   3.71417   5.45437   0.354784
-8.27102  -8.55020   9.1397   5.45437   8.50561   0.586322
-0.67588  -0.70540   0.6580   0.35478   0.58632   0.238650
```

Model L3

Ordinal Logistic Regression: Score versus logC, Time

```
Link Function: Normit

Response Information

Variable  Value  Count
Score     0         11
          1          9
          2         17
          Total     37
```

```
Factor Information

Factor  Levels  Values
Time         3  10, 60, 180

* NOTE * 37 cases were used
* NOTE * 1 cases contained missing values

[DRS] Female Pig 63 is an outlier and was dropped from this analysis.

Logistic Regression Table

Predictor      Coef  SE Coef      Z      P
Const(1)    8.31350  2.31076   3.60  0.000
Const(2)    9.21873  2.38045   3.87  0.000
logC       -10.2284  2.65272  -3.86  0.000
Time
  60       -6.07721  1.66820  -3.64  0.000
 180       -9.31643  2.44364  -3.81  0.000

Tests for terms with more than 1 degree of freedom

Term  Chi-Square  DF      P
Time     14.5493   2  0.001

Log-Likelihood = -29.778
Test that all slopes are zero: G = 19.019, DF = 3, P-Value = 0.000

Goodness-of-Fit Tests

Method     Chi-Square  DF      P
Pearson       77.7416  67  0.174
Deviance      59.5555  67  0.729

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs       Number  Percent  Summary Measures
Concordant     359     81.8  Somers' D              0.64
Discordant      80     18.2  Goodman-Kruskal Gamma  0.64
Ties             0      0.0  Kendall's Tau-a        0.42
Total          439    100.0

MTB > print m4
```

Data Display

```
 Matrix XPWX6

 5.33961   5.46658  -6.04159  -3.75441  -5.56947
 5.46658   5.66654  -6.23213  -3.86714  -5.74398
-6.04159  -6.23213   7.03695   4.21734   6.33423
-3.75441  -3.86714   4.21734   2.78289   3.92894
-5.56947  -5.74398   6.33423   3.92894   5.97139
```

Model L4

Ordinal Logistic Regression: Score versus logC, logT, sex

```
Link Function: Normit
```

```
Response Information

Variable  Value  Count
Score     0         11
          1          9
          2         17
          Total     37

Factor Information

Factor  Levels  Values
sex          2  female, male

* NOTE * 37 cases were used
* NOTE * 1 cases contained missing values

[DRS] Female Pig 63 is an outlier and was dropped from this analysis.

Logistic Regression Table

Predictor       Coef   SE Coef      Z      P
Const(1)     22.2598   5.85398   3.80  0.000
Const(2)     23.3509   5.95337   3.92  0.000
logC        -13.5029   3.42349  -3.94  0.000
logT        -10.2976   2.68506  -3.84  0.000
sex
 male       -3.06349   1.72433  -1.78  0.076
sex*logT
 male        1.07283  0.930951   1.15  0.249

Log-Likelihood = -25.921
Test that all slopes are zero: G = 26.733, DF = 4, P-Value = 0.000

Goodness-of-Fit Tests

Method    Chi-Square  DF      P
Pearson      55.4602  66  0.819
Deviance     51.8415  66  0.899

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs       Number  Percent  Summary Measures
Concordant     377     85.9  Somers' D              0.72
Discordant      62     14.1  Goodman-Kruskal Gamma  0.72
Ties             0      0.0  Kendall's Tau-a        0.47
Total          439    100.0

MTB > print m5
```

Data Display

```
 Matrix XPWX7

 34.2690   34.8050  -19.7042  -15.6628  -5.67531   2.46103
 34.8050   35.4426  -20.0507  -15.9283  -5.75225   2.48795
-19.7042  -20.0507   11.7203    8.9509   2.70952  -1.09640
-15.6628  -15.9283    8.9509    7.2096   2.68421  -1.21099
 -5.6753   -5.7523    2.7095    2.6842   2.97331  -1.53557
  2.4610    2.4879   -1.0964   -1.2110  -1.53557   0.86667
```

Model L5

Ordinal Logistic Regression: Score versus logC, logT, sex

```
Link Function: Normit

Response Information

Variable  Value  Count
Score     0         11
          1          9
          2         17
          Total     37

Factor Information

Factor  Levels  Values
sex          2  female, male

* NOTE * 37 cases were used
* NOTE * 1 cases contained missing values

[DRS] Female Pig 63 is an outlier and was dropped from this analysis.

Logistic Regression Table

Predictor      Coef    SE Coef      Z      P
Const(1)    19.6521    5.07896   3.87  0.000
Const(2)    20.6991    5.17367   4.00  0.000
logC       -12.4093    3.12741  -3.97  0.000
logT        -8.97914   2.28809  -3.92  0.000
sex
 male       -1.21073   0.489222 -2.47  0.013

Log-Likelihood = -26.640
Test that all slopes are zero: G = 25.296, DF = 3, P-Value = 0.000

Goodness-of-Fit Tests

Method    Chi-Square  DF      P
Pearson      60.1095  67  0.712
Deviance     53.2794  67  0.889

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs       Number  Percent  Summary Measures
Concordant     374     85.2  Somers' D              0.70
Discordant      65     14.8  Goodman-Kruskal Gamma  0.70
Ties             0      0.0  Kendall's Tau-a        0.46
Total          439    100.0

MTB > print m1
```

Data Display

```
 Matrix XPWX3

 25.7958   26.2335  -15.6902  -11.5781  -1.18443
 26.2335   26.7668  -15.9878  -11.7947  -1.21315
-15.6902  -15.9878    9.7807    7.0269   0.68225
```

```
-11.5781  -11.7947    7.0269    5.2353   0.48445
 -1.1844   -1.2131    0.6823    0.4845   0.23934
```

Model L6

Ordinal Logistic Regression: Score versus logC, logT

```
Link Function: Normit

Response Information

Variable  Value  Count
Score     0         11
          1          9
          2         17
          Total     37

* NOTE * 37 cases were used
* NOTE * 1 cases contained missing values

[DRS] Female Pig 63 is an outlier and was dropped from this analysis.

Logistic Regression Table

Predictor      Coef  SE Coef      Z      P
Const(1)    15.1659  4.05345   3.74  0.000
Const(2)    16.0604  4.12562   3.89  0.000
logC        -9.88150  2.54186  -3.89  0.000
logT        -7.22086  1.88084  -3.84  0.000

Log-Likelihood = -30.001
Test that all slopes are zero: G = 18.572, DF = 2, P-Value = 0.000

Goodness-of-Fit Tests

Method    Chi-Square  DF      P
Pearson      79.7589  68  0.156
Deviance     60.0028  68  0.744

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs       Number  Percent  Summary Measures
Concordant     352     80.2  Somers' D              0.61
Discordant      86     19.6  Goodman-Kruskal Gamma  0.61
Ties             1      0.2  Kendall's Tau-a        0.40
Total          439    100.0

MTB > print m6
```

Data Display

```
 Matrix XPWX8

 16.4305   16.6894  -10.1366  -7.59962
 16.6894   17.0207  -10.3226  -7.73621
-10.1366  -10.3226    6.4611   4.66624
 -7.5996   -7.7362    4.6662   3.53758
```

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